# IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Patentee: | Radin *et al.* | **Certificate Under 37 CFR 1.10**<br>I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as express mail, Express Mail No. EV 417 059 006 US in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450<br><br>on June 26, 2012<br><br>(Signature)<br><br>Eric E. Williams<br>(Printed Name) |
| U.S. Patent No. | 5,929,304 | |
| Issue Date: | July 27, 1999 | |
| Title: | Production of Lysosomal Enzymes in Plant-Based Expression Systems | |
| Assignees: | Virginia Tech Intellectual Properties, Inc. *et al.* | |
| Attorney Docket No. | 55820-49767 | |

## REQUEST FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. § 156

Mail Stop Hatch-Waxman PTE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Pursuant to Section 201(a) of the Drug Price Competition and Patent Term Restoration Act of 1984, 35 U.S.C. § 156, Virginia Tech Intellectual Properties, Inc. (hereinafter "VTIP"), an owner of the above-identified patent, hereby requests an extension of the patent term of U.S. Patent No. 5,929,304. Applicant VTIP is an owner of U.S. Patent No. 5,929,304 according to the following chain of title.

1. Assignment of all rights of inventor Carole L. Cramer to Virginia Polytechnic Institute and State University, by virtue of an Assignment recorded in the U.S. Patent and Trademark Office on January 24, 1997, at Reel 008324, Frame 0288.

2. Assignment of all rights of Virginia Polytechnic Institute and State University to Virginia Tech Intellectual Properties, Inc., by virtue of an Assignment recorded in the U.S. Patent and Trademark Office on January 24, 1997, at Reel 008324, Frame 0304.

The U.S. Patent and Trademark Office Patent Assignment Abstract of Title for U.S. Patent No. 5,929,304 is attached hereto as Exhibit A.

For full disclosure, Exhibit A shows that the captioned patent has two assignees. The first assignee is VTIP. The second assignee is Croptech Development Corporation.

The following information is submitted in accordance with 35 U.S.C. § 156(d) and 37 C.F.R. § 1.710 *et seq.* and follows the numerical format set forth in 37 C.F.R. § 1.740(a):

**(1) A complete identification of the approved product as by appropriate chemical and generic name, physical structure or characteristics:**

The approved product is taliglucerase alfa, a hydrolytic lysosomal glucocerebroside-specific enzyme. Taliglucerase alfa is a recombinant active form of the lysosomal enzyme, *β*-glucocerebrosidase, which is expressed in genetically modified carrot plant root cells cultured in a disposable bioreactor system (ProCellEx®). *β*-Glucocerebrosidase (*β*-D-glucosyl-N-acylsphingosine glucohydrolase) is a lysosomal glycoprotein enzyme that catalyzes the hydrolysis of the glycolipid glucocerebroside to glucose and ceramide. Taliglucerase alfa is produced by recombinant DNA technology using plant cell culture (*i.e.*, carrot). Purified taliglucerase alfa is a monomeric glycoprotein containing four (4) N-linked glycosylation sites (Mr = 60,800). Taliglucerase alfa differs from native human glucocerebrosidase by two amino acids at the N terminal and up to seven (7) amino acids at the C terminal. Taliglucerase alfa is a glycosylated protein with oligosaccharide chains at the glycosylation sites having terminal mannose sugars. These mannose-terminated oligosaccharide chains of taliglucerase alfa are specifically recognized by endocytic carbohydrate receptors on macrophages, the cells that accumulate lipid in Gaucher disease.

Taliglucerase alfa is the active ingredient in the product ELELYSO™ as may be seen from attached Exhibit B, which is a copy of the labeling information for the approved product.

**(2) A complete identification of the Federal statute including the applicable provision of law under which the regulatory review occurred:**

The regulatory review occurred under Section 505 of the Federal Food, Drug, and Cosmetic Act (FFDCA), 21 U.S.C. § 301 *et seq.* Section 505 provides for the submission and approval of new drug applications (NDAs) for drug products meeting the definition of "new drug" under Section 201(p) of the Act.

**(3) An identification of the date on which the product received permission for commercial marketing or use under the provision of law under which the applicable regulatory review period occurred:**

Taliglucerase alfa was approved by the Food and Drug Administration (FDA) for commercial marketing pursuant to Section 505 of the FFDCA on May 1, 2012.

**(4) In the case of a drug product, an identification of each active ingredient in the product and as to each active ingredient, a statement that it has not been previously approved for commercial marketing or use under the Federal Food, Drug, and Cosmetic Act, the Public Health Service Act, or the Virus-Serum-Toxin Act, or a statement of when the active ingredient was approved for commercial marketing or use (either alone or in combination with other active ingredients), the use for which it was approved, and the provision of law under which it was approved:**

As stated in Sections 1, 2, and 3 above, the active ingredient in the product ELELYSO™ is taliglucerase alfa. Taliglucerase alfa was not previously approved for commercial marketing or use under Section 505 of the FFDCA, the Public Health Service Act, or the Virus-Serum-Toxin Act. Taliglucerase alfa was approved for injection as a hydrolytic lysosomal glucocerebroside-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for adults with a confirmed diagnosis of Type 1 Gaucher disease under Section 505 of the FFDCA on May 1, 2012.

**(5) A statement that the application is being submitted within the sixty day period permitted for submission pursuant to § 1.720(f) and an identification of the date of the last day on which the application could be submitted:**

The product was approved on May 1, 2012 and the sixty-day period permitted for submission of an application for extension of a patent runs through June 29, 2012. Accordingly, this application is timely filed.

**(6) A complete identification of the patent for which an extension is being sought by the name of the inventor, the patent number, the date of issue, and the date of expiration:**

| | |
|---|---|
| Inventors: | David N. Radin, Carole L. Cramer, Karen K. Oishi, and Deborah L. Weissenborn |
| U.S. Patent No.: | 5,929,304 |
| Issue Date: | July 27, 1999 |
| Expiration Date: | September 13, 2016 |

**(7) A copy of the patent for which an extension is being sought, including the entire specification (including claims) and drawings:**

A copy of the patent is attached as Exhibit C.

**(8) A copy of any disclaimer, certificate of correction, receipt of maintenance fee payment, or reexamination certificate issued in the patent:**

No maintenance fees are outstanding for this patent. A statement evidencing the three maintenance fee payments for the patent is enclosed as Exhibit D.

On June 19, 2012, a change in entity status was submitted for the captioned patent along with the difference between the small entity fee paid and large entity fee due for the 12th year maintenance fee. A copy of the submission regarding the change in entity status and the receipt showing the payment of the large entity fee is included in Exhibit D.

**(9) A statement that the patent claims the approved product, or a method of using or manufacturing the approved product, and a showing which lists each applicable patent claim and demonstrates the manner in which at least one such patent claim reads on: (i) The approved product, if the listed claims include any claim to the approved product; (ii) The method of using the approved product, if the listed claims include any claim to the method of using the approved product; and (iii) The method of manufacturing the approved product, if the listed claims include any claim to the method of manufacturing the approved product:**

U.S. Patent No. 5,929,304 claims, among other subject matter, at least methods of manufacturing the approved product, which is taliglucerase alfa.

The applicable claims are at least claims 1, 6, 11-13, 15-17, 19, 23, 26-32, 34-36, 40, 44, 47-49, 51-53, 59, 63, 66-68, and 70-72. In addition, some of the other claims may be applicable, such as claims 7-10, 18, 24-25, 33, 37-39, 45-46, 54-58, 64-65, and 73.

In general terms, claims 1, 6-13, and 15-18 recite a method for producing a lysosomal enzyme which is enzymatically active; claims 19 and 23-39 recite a recombinant expression construct; claims 40, 44-49, and 51-58 recite a transgenic plant or plant cell capable of producing a lysosomal enzyme which is enzymatically active; and claims 59, 63-68, and 70-73 recite a lysosomal enzyme which is enzymatically active and is produced according to a stated process. The manner in which claim 1 reads on the approved product is as follows:

<u>**Claim 1:**</u>

Claim 1 recites the following:

> A method for producing a lysosomal enzyme which is enzymatically active, comprising:
>
> recovering the lysosomal enzyme from (i) a transgenic plant cell or (ii) a cell, tissue or organ of a transgenic plant, which transgenic plant cell or plant is transformed or transfected with a recombinant expression construct comprising a nucleotide sequence encoding the lysosomal enzyme and a promoter that regulates expression of the nucleotide sequence so that the lysosomal enzyme is expressed

by the transgenic plant cell or plant.

Claim 1 reads on the approved product because the active ingredient of the approved product, taliglucerase alfa, is a lysosomal enzyme which is enzymatically active and is produced via recovery from a transgenic carrot cell according to the claimed method.

**(10) A statement beginning on a new page of the relevant dates and information pursuant to 35 U.S.C. §156(g) in order to enable the Secretary of Health and Human Services or the Secretary of Agriculture, as appropriate, to determine the applicable regulatory review period as follows:**

**(i) For a patent claiming a human drug, antibiotic, or human biological product: (A) The effective date of the investigational new drug (IND) application and the IND number; (B) The date on which a new drug application (NDA) or a Product License Application (PLA) was initially submitted and the NDA or PLA number; and (C) The date on which the NDA was approved or the Product License issued;**

**(ii) For a patent claiming a new animal drug: (A) The date a major health or environmental effects test on the drug was initiated, and any available substantiation of that date, or the date of an exemption under subsection (j) of Section 512 of the Federal Food, Drug, and Cosmetic Act became effective for such animal drug; (B) The date on which a new animal drug application (NADA) was initially submitted and the NADA number; and (C) The date on which the NADA was approved;**

**(iii) For a patent claiming a veterinary biological product: (A) The date the authority to prepare an experimental biological product under the Virus-Serum-Toxin Act became effective; (B) The date an application for a license was submitted under the Virus-Serum-Toxin Act; and (C) The date the license issued;**

**(iv) For a patent claiming a food or color additive: (A) The date a major health or environmental effects test on the additive was initiated and any available substantiation of that date; (B) The date on which a petition for product approval under the Federal Food, Drug, and Cosmetic Act was initially submitted and the petition number; and (C) The date on which the FDA published a *Federal Register* notice listing the additive for use;**

**(v) For a patent claiming a medical device: (A) The effective date of the investigational device exemption (IDE) and the IDE number, if applicable, or the date on which the applicant began the first clinical investigation involving the device, if no IDE was submitted, and any available substantiation of that date; (B) The date on which the application for product approval or notice of completion of a product development protocol under Section 515 of the Federal Food, Drug, and Cosmetic Act was initially submitted and the number of the application; and (C) The date on which the application was approved or the protocol declared to be completed:**

According to the provisions of 37 C.F.R. § 1.740(10)(i), the relevant dates and information to enable the Secretary of Health and Human Services to determine the applicable regulatory review period are as follows:

(A) On June 16, 2005, Protalix Ltd., a licensee to the above-captioned patent, submitted Investigational New Drug (IND) No. 069703 (plant cell expressed recombinant human glucocerobrosidase (taliglucerase alfa)) to the FDA to permit interstate shipment of taliglucerase alfa for the purpose of conducting clinical studies to support the approval of a subsequent NDA for taliglucerase alfa. The FDA acknowledged receipt of and accepted the IND on June 16, 2005. According to Subsection (i)(2) of Section 505 of the FFDCA, clinical investigation of a new drug may begin thirty days after the FDA's receipt of the IND application from the sponsor, provided that the FDA does not determine a clinical hold is necessary. A clinical hold was issued for IND No. 069703 on July 15, 2005. The clinical hold was lifted on April 16, 2007. Accordingly, the IND became effective on the date the clinical hold was lifted. This establishes the beginning of the "regulatory review period" under 35 U.S.C. § 156(g)(1) as April 16, 2007, the effective date of an exemption under Section 505(i). A copy of an FDA Review, which includes a summary of the IND regulatory submissions, is attached as Exhibit E.

(B) On April 26, 2010, Protalix Ltd. submitted the final section of an NDA under a rolling review for taliglucerase alfa, NDA No. 022458. The NDA final submissions were received by the FDA on April 26, 2010 as indicated by Exhibit F. Thus, for the purposes of the "regulatory review period" under 35 U.S.C. § 156(g)(1), April 26, 2010 is the date of initial submission of a new drug application under Section 505 for taliglucerase alfa.

(C) NDA No. 022458 was approved on May 1, 2012. Attached as Exhibit G is a letter dated May 1, 2012 from the FDA to Protalix Ltd. regarding approval of the NDA for taliglucerase alfa. Thus, for the purposes of the "regulatory review period" under 35 U.S.C. § 156(g)(1), May 1, 2012 is the date of approval of the new drug application for taliglucerase alfa submitted on April 26, 2010.

**(11) A brief description beginning on a new page of the significant activities undertaken by the marketing applicant during the applicable regulatory review period with respect to the approved product and the significant dates applicable to such activities:**

During the applicable regulatory review period, Protalix Ltd., a licensee to the above-captioned patent, was actively involved in obtaining NDA approval for taliglucerase alfa. As discussed in paragraph (10) above, the IND for taliglucerase alfa was submitted on June 16, 2005, the clinical hold on the IND was lifted on April 16, 2007, the NDA was submitted on April 26, 2010, and the NDA was approved on May 1, 2012. Protalix Ltd. was in consultation with the FDA during the clinical studies conducted under the IND. Similarly, subsequent to the submission of the NDA, Protalix Ltd. had numerous contacts and meetings with the FDA with respect to the approval and, in fact, conducted additional studies at FDA's request to support the NDA approval. The description of significant activities undertaken by Protalix Ltd. with respect to taliglucerase alfa during the regulatory review period is set forth in Exhibit H and is illustrative of the activities involved.

**(12) A statement beginning on a new page that in the opinion of the applicant the patent is eligible for the extension and a statement as to the length of extension claimed, including how the length of extension was determined:**

(a) Statement of eligibility of the patent for extension under 35 U.S.C. § 156(a):

Section 156(a) provides, in relevant part, that the term of a patent which claims a product, a method of using a product, or a method of manufacturing a product shall be extended if (1) the term of the patent has not expired before an application for extension is submitted, (2) the term of the patent has never been extended, (3) the application for extension is submitted by the owner of record of the patent or its agent and in accordance with 35 U.S.C. § 156(d), (4) the product has been subject to a regulatory review period before its commercial marketing or use, and (5) the permission for the commercial marketing or use of the product after such regulatory review period is the first permitted commercial marketing or use of the product under the provision of law under which such regulatory review period occurred.

As described below by corresponding number, each of these elements is satisfied here:

(1) The term of U.S. Patent No. 5,929,304 expires on September 13, 2016. This application has, therefore, been submitted before the expiration of the patent term.

(2) The term of this patent has never been extended.

(3) This application is submitted by an owner of record, Virginia Tech Intellectual Properties, Inc. (according to the assignments recorded in the U.S. Patent and Trademark Office on January 24, 1997, at Reel 008324, Frame 0288 and at Reel 008324, Frame 0304). This application is submitted in accordance with 35 U.S.C. § 156(d) in that it is submitted within the sixty-day period beginning on the date, May 1, 2012, the product received permission for marketing under the FFDCA, and contains the information required under 35 U.S.C. § 156(d).

(4) As evidenced by the May 1, 2012 letter from the FDA (Exhibit G), the product was subjected to a regulatory review period under Section 505 of the FFDCA before its commercial marketing or use.

(5)(a) The permission for the commercial marketing of taliglucerase alfa after regulatory review under Section 505 is the first permitted commercial marketing of taliglucerase alfa. This is confirmed by the absence of any new drug application approved under Section 505 for taliglucerase alfa prior to May 1, 2012.

(5)(b) Finally, the permission for the commercial marketing or use of taliglucerase alfa after regulatory review under Section 505 is the first permitted commercial marketing or use of taliglucerase alfa under the process and/or method of manufacturing taliglucerase alfa claimed in the patent. This is confirmed by the absence of any new drug application approved under Section 505 for taliglucerase alfa prior to May 1, 2012

(b) Statement as to length of extension claimed:

The term of U.S. Patent No. 5,929,304 should be extended by 1291 days to March 27, 2020. This extension was determined on the following basis: as set forth in 35 U.S.C. § 156(g)(1) and 37 C.F.R. § 1.775(c), the regulatory review period equals the length of time between the effective date of the IND on April 16, 2007 and the initial submission of the NDA on April 26, 2010, a period of 1107 days, plus the length of time between the initial submission of the NDA on April 26, 2010 to NDA approval on May 1, 2012, a period of 737 days. These two periods added together equal 1844 days.

Pursuant to 35 U.S.C. § 156(c) and 37 C.F.R. § 1.775 (d)(1)(i), the term of the patent eligible for extension shall be extended by the time equal to the regulatory review period which occurs after the date the patent was issued. The entire period under 35 U.S.C. § 156(g)(1)(B) occurred after the July 27, 1999 issue date of U.S. Patent No. 5,929,304. Thus, the 1844-day period calculated above as the term of the patent eligible for extension should not be reduced under 35 U.S.C. § 156(c) or 37 C.F.R. § 1.775(d)(1)(i).

As discussed in paragraph (11) above and as illustrated in Exhibit H, it is believed that Protalix Ltd., a licensee to the above-captioned patent, was continuously working toward securing NDA approval for taliglucerase alfa. As it is believed Protalix Ltd. acted with appropriate due diligence during the period of regulatory review, the 1844-day period calculated above as the term of the patent eligible for extension should not be reduced for lack of diligence under 35 U.S.C. § 156(c)(1) or 37 C.F.R. § 1.775(d)(1)(ii).

Pursuant to 35 U.S.C. § 156(c)(2) and 37 C.F.R. § 1.775(d)(1)(iii), this 1844-day period is to be reduced by one-half of the time from the effective date of the IND (April 16, 2007), or the date of issue of U.S. Patent No. 5,929,304 (July 27, 1999), whichever is later, to the date of submission of the NDA, April 26, 2010, a period of 1107 days. One-half of this period is 553.5 days. According to MPEP § 2758, "half days will be ignored and thus will

not be subtracted from the regulatory review period." Thus, the 1844-day period is reduced by 553 days, resulting in a revised regulatory period of 1291 days.

Pursuant to 35 U.S.C. § 156(c)(3) and 37 C.F.R. § 1.775(d)(2-4), if the period remaining in the term of the patent after the date of approval May 1, 2012 to September 13, 2016, a period of 1596 days, when added to the revised regulatory period (1291 days) exceeds 14 years (5113 days), the period of extension must be reduced so that the total of both such periods does not exceed fourteen years. In this case, the total of both such periods does not exceed fourteen years and, therefore, the 1291-day revised regulatory review period is not reduced.

The period of patent term extension as calculated above is also subject to the provisions of 35 U.S.C. § 156(g)(6) and 37 C.F.R. § 1.775(d)(5). U.S. Patent No. 6,063,771 issued after the enactment of the statute, September 24, 1984 and, thus, the five-year maximum on extension as provided in 35 U.S.C. § 1.56(g)(6) and 37 C.F.R. § 1.775(d)(5) is applicable. Since this five-year maximum period is greater than the period calculated above, the term of the patent is eligible for a 1291-day extension until March 27, 2020.

**(13) A statement that applicant acknowledges a duty to disclose to the Commissioner for Patents and the Secretary of Health and Human Services or the Secretary of Agriculture any information which is material to the determination of entitlement to the extension sought (*see* § 1.765):**

Applicant acknowledges a duty to disclose to the Commission for Patents and the Secretary of Health and Human Services any information which is material to any determination of entitlement to the extension sought. Applicant is unaware of any such information other than that already presented in this application and attached Exhibits.

**(14) The prescribed fee for receiving and acting upon the application for extension (see § 1.20(j)):**

As indicated by the letter of transmittal submitted with this application, the Commissioner for Patents has been authorized to charge the filing fee of $1,120.00 and any

additional fees which may be required by this or any other related paper, or credit any overpayment or any additional fees which may be required, to Deposit Account No. 10-0435 in the name of Barnes & Thornburg LLP, with reference to our matter number 55820-49767.

**(15) The name, address, and telephone number of the person to whom inquiries and correspondence relating to the application for patent term extension are to be directed:**

James J. Sales
Barnes & Thornburg LLP
11 S. Meridian Street
Indianapolis, IN 46204
(317) 231-6423

**********

Pursuant to 37 C.F.R. § 1.740(b) and MPEP § 2753, two additional copies of this application (for a total of three copies) are submitted herewith.

As the undersigned agent of VTIP, an owner of record of U.S. Patent No. 5,929,304, which, by submission of this paper and attached Exhibits, now applies for an extension of term of this patent, I, James J. Sales, declare that (1) I am a Patent Attorney authorized to practice before the Patent and Trademark Office and have general authority from VTIP to act on its behalf on at least this patent matter; that (2) I have reviewed and understand the contents of this application for extension of U.S. Patent No. 5,929,304; that (3) I believe the patent is subject to extension pursuant to 37 C.F.R. § 1.714; that (4) I believe the length of extension claimed is fully justified under 35 U.S.C. § 156 and applicable regulations; and that (5) I believe the patent for which this extension is being sought meets the conditions for extension of the term of a patent as set forth in 37 C.F.R. § 1.720.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and, further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent extension issuing thereon.

Respectfully submitted,

/James J. Sales/
James J. Sales
Attorney Reg. No. 33,773

# EXHIBIT A

United States Patent and Trademark Office



Assignments on the Web > **Patent Query**

# Patent Assignment Abstract of Title

***NOTE:Results display only for issued patents and published applications. For pending or abandoned applications please consult USPTO staff.***

**Total Assignments: 3**

**Patent #:** 5929304 **Issue Dt:** 07/27/1999 **Application #:** 08713928 **Filing Dt:** 09/13/1996

**Inventors:** DAVID N. RADIN, CAROLE L. CRAMER, KAREN K. OISHI, DEBORAH L. WEISSENBORN

**Title:** PRODUCTION OF LYSOSOMAL ENZYMES IN PLANT-BASED EXPRESSION SYSTEMS

**Assignment: 1**

**Reel/Frame:** 008324/0288 **Recorded:** 01/24/1997 **Pages:** 2

**Conveyance:** ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS).

**Assignor:** CRAMER, CAROLE L. **Exec Dt:** 11/07/1996

**Assignee:** VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY
306 BURRUS HALL
BLACKSBURG, VIRGINIA 24061

**Correspondent:** PENNIE & EDMONDS
GEORGE C. JEN
1667 K STREET, N.W.
WASHINGTON, D.C. 20006

**Assignment: 2**

**Reel/Frame:** 008324/0635 **Recorded:** 01/24/1997 **Pages:** 3

**Conveyance:** ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS).

**Assignors:** RADIN, DAVID N. **Exec Dt:** 11/08/1996
OISHI, KAREN K. **Exec Dt:** 11/08/1996
WEISSENBORN, DEBORAH L. **Exec Dt:** 11/08/1996

**Assignee:** CROPTECH DEVELOPMENT CORPORATION
1861 PRATT DRIVE, SUITE 1040
BLACKSBURG, VIRGINIA 24060

**Correspondent:** PENNIE & EDMONDS
GEORGE C. JEN
1667 K STREET, N.W.
WASHINGTON, D.C. 20006

**Assignment: 3**

**Reel/Frame:** 008324/0304 **Recorded:** 01/24/1997 **Pages:** 2

**Conveyance:** ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS).

**Assignor:** VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIV. **Exec Dt:** 01/06/1997

**Assignee:** VIRGINIA TECH INTELLECTUAL PROPERTIES, INC.
1900 KRAFT DRIVE, SUITE 107
BLACKSBURG, VIRGINIA 24060

**Correspondent:** PENNIE & EDMOUNDS
GEORGE C. JEN
1667 K STREET, N.W.
WASHINGTON, D.C. 20006

EXHIBIT A

PENGAD 800-631-6989

Search Results as of: 06/14/2012 04:58 PM

# EXHIBIT B

**HIGHLIGHTS OF PRESCRIBING INFORMATION**
**These highlights do not include all the information needed to use ELELYSO safely and effectively. See full prescribing information for ELELYSO.**

**ELELYSO (taliglucerase alfa) for injection, for intravenous use**
**Initial US Approval: 2012**

**—INDICATIONS AND USAGE—**

ELELYSO™ (taliglucerase alfa) for injection is a hydrolytic lysosomal glucocerebroside-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for adults with a confirmed diagnosis of Type 1 Gaucher disease. (1)

**—DOSAGE AND ADMINISTRATION—**

- 60 Units/kg administered every other week as a 60-120 minute intravenous infusion (2.1)
- Patients currently being treated with imiglucerase for Gaucher disease can be switched to ELELYSO. Patients previously treated on a stable dose of imiglucerase are recommended to begin treatment with ELELYSO at that same dose when they switch from imiglucerase to ELELYSO (2.1).
- Physicians can make dosage adjustments based on achievement and maintenance of each patient's therapeutic goals. Clinical trials have evaluated doses ranging from 11 Units/kg to 73 Units/kg every other week (2).

**—DOSAGE FORMS AND STRENGTHS—**

- For injection: lyophilized powder for reconstitution with diluent (3).
- Available in 200 Unit single-use vials (3)

**—CONTRAINDICATIONS—**

None (4)

**—WARNINGS AND PRECAUTIONS—**

- Anaphylaxis: Anaphylaxis has been observed in some patients treated with ELELYSO. If anaphylaxis occurs, immediately discontinue infusion and initiate appropriate treatment (5.1).
- Allergic and Infusion Reactions: The most commonly observed symptoms of infusion reactions (including allergic reactions) were headache, chest pain or discomfort, asthenia, fatigue, urticaria, erythema, increased blood pressure, back pain, arthralgia, and flushing. If allergic or infusion reactions occur, decreasing the infusion rate, temporarily stopping the infusion, or administering antihistamines and/or antipyretics is recommended (5.2).

**—ADVERSE REACTIONS—**

The most common adverse reactions during clinical studies were infusion reactions (6.1). Other commonly observed adverse reactions in ≥10% of patients were URTI/nasopharyngitis, pharyngitis/throat infection, headache, arthralgia, influenza/flu, UTI/pyelonephritis, back pain, extremity pain(6.1).

**To report SUSPECTED ADVERSE REACTIONS, contact Pfizer Inc at (1-800-438-1985) or FDA at 1-800-FDA-1088 or *www.fda.gov/medwatch*.**

**See 17 for PATIENT COUNSELING INFORMATION.**

**Revised: May 2012**

EXHIBIT
B
PENGAD 800-631-6989

Reference ID: 3124792

## FULL PRESCRIBING INFORMATION: CONTENTS*



*Sections or subsections omitted from the full prescribing information are not listed

**FULL PRESCRIBING INFORMATION**

## 1 INDICATIONS AND USAGE

ELELYSO™ (taliglucerase alfa) for injection is a hydrolytic lysosomal glucocerebroside-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for adults with a confirmed diagnosis of Type 1 Gaucher disease.

## 2 DOSAGE AND ADMINISTRATION

### 2.1 Recommended Dose

The recommended dose is 60 Units/kg of body weight administered once every 2 weeks as a 60-120 minute intravenous infusion.

Patients currently being treated with imiglucerase for Type 1 Gaucher disease can be switched to ELELYSO. Patients previously treated on a stable dose of imiglucerase are recommended to begin treatment with ELELYSO at that same dose when they switch from imiglucerase to ELELYSO.

Dosage adjustments can be made based on achievement and maintenance of each patient's therapeutic goals. Clinical studies have evaluated dose ranges from 11 Units/kg to 73 Units/kg every other week.

ELELYSO should be reconstituted with Sterile Water for Injection and diluted with 0.9% Sodium Chloride Injection, USP, to a final volume of 100mL to 200 mL, and delivered by intravenous infusion. The initial infusion rate should be 1.3 mL/min. After patient tolerability to the infusion rate is established, the rate of infusion may be increased to 2.3 mL/min. The total volume of the infusion solution should be delivered over a period of no less than 1 hour.

Each vial of ELELYSO provides 200 Units of taliglucerase alfa and is intended for single use only. Do not use the vial more than one time. The reconstitution and dilution steps must be completed using aseptic techniques. ELELYSO should be prepared using low-protein-binding containers and administered with a low-protein-binding infusion set equipped with an in-line, low-protein-binding 0.2 micrometer filter.

### 2 2 Instructions for Use

ELELYSO should be reconstituted, diluted, and administered under the supervision of a healthcare professional.

Prepare and use ELELYSO according to the following steps. Use aseptic technique.

a. Determine the number of vials to be reconstituted based on the patient's weight and the recommended dose of 60 Units/kg, using the following calculations (1-3):

   (1) Total dose in Units = Patient's weight (kg) x 60 Units/kg

   (2) Total number of vials = Total dose in Units divided by 200 Units/vial

   (3) Round up to the next whole vial.

b. Remove the required number of vials from the refrigerator. Do not leave these vials at room temperature longer than 24 hours prior to reconstitution. Do not heat or microwave these vials.

c. Reconstitute each vial of ELELYSO with 5.1 mL of Sterile Water for Injection to yield a reconstituted product volume of 5.3 mL and a withdrawal volume of 5 mL. Upon reconstitution, mix vials gently. DO NOT SHAKE. Prior to further dilution, visually inspect the solution in the vials; the solution should be clear and colorless. Do not use if the solution is discolored or if foreign particulate matter is present.

d. Withdraw 5 mL of reconstituted solution from each vial and dilute with 0.9% Sodium Chloride Injection, USP, to a final volume of 100 – 200 mL. Mix gently. DO NOT SHAKE. Since this is a protein solution, slight flocculation (described as translucent fibers) occurs occasionally after dilution.

As ELELYSO contains no preservative, the product should be used immediately once reconstituted. If immediate use is not possible, the reconstituted or the diluted product may be stored for up to 24 hours at 2 to 8°C (36 to 46 F). Do not freeze. Protect from light. Discard any unused product.

## 3 DOSAGE FORMS AND STRENGTHS

For injection: lyophilized powder for reconstitution; 200 Units/vial.

## 4 CONTRAINDICATIONS

None.

## 5 WARNINGS AND PRECAUTIONS

### 5.1 Anaphylaxis

As with any intravenous protein product, severe allergic reactions are possible. Anaphylaxis has been reported in patients treated with ELELYSO *[see Adverse Reactions (6.1)]*. If anaphylaxis occurs, ELELYSO should be immediately discontinued, and appropriate medical treatment should be initiated.

In patients who have experienced anaphylaxis during infusion with ELELYSO, caution should be exercised upon rechallenge; appropriate medical support should be readily available *[see Adverse Reactions (6)]*.

### 5.2 Allergic and Infusion Reactions

Infusion reactions (including allergic reactions), defined as a reaction occurring within 24 hours of the infusion, were the most commonly observed reactions in patients (44%-46%) treated with ELELYSO in clinical studies *[see Adverse Reactions (6)]*. The most commonly observed symptoms of infusion reactions were headache (16%), chest pain or discomfort (6%), asthenia (7%), fatigue (5%), urticaria (7%), erythema (5%), increased blood pressure (5%), back pain and arthralgia (7%), and flushing (6%)._ Other infusion or allergic reactions reactions included, angioedema, wheezing, dyspnea, coughing, cyanosis, and hypotension. Most of these reactions were mild and did not require treatment intervention.

Base the management of infusion reactions on the type and severity of the reaction, e.g., slowing the infusion rate or treatment with medications such as antihistamines and antipyretics.

Pre-treatment with antihistamines and/or corticosteroids may prevent subsequent reactions in those cases where symptomatic treatment was required. Patients were not routinely pre-medicated prior to infusion of ELELYSO during clinical studies.

## 6 ADVERSE REACTIONS

### 6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

The data described below reflect exposure to ELELYSO in 60 patients ages 13 to 74 years who received ELELYSO at doses ranging from 11 to 73 Units/kg every two weeks in 3 clinical studies, and included 31 males and 29 females. Thirty-two patients were naïve to ERT (Study 1) and 28 were switched from imiglucerase to ELELYSO (Study 2) *[see Clinical Studies (14)]*. Study 3 includes patients continuing treatment from Study 1 and Study 2. Twenty-four patients were treated for longer than 2 years and 4 patients were treated longer than 3 years.

Important adverse reactions including anaphylaxis, allergic reactions, and infusion reactions are described elsewhere in the label *[see Warnings and Precautions (5.1)]*. One patient experienced a Type III immune-mediated skin reaction. The most common adverse reactions requiring interventions were infusion reactions *[see Warnings and Precautions (5.2)]*.

Table 2 is a listing of adverse reactions that occurred in 10% or greater of patients.

Table 2: Adverse Reactions that Occurred in ≥10% of Patients Treated with ELELYSO

| | Study 1 | Study 2 |
|---|---|---|
| Preferred Term | N=32 | N=28 |
| Infusion reaction | 14 (44%) | 13 (46%) |
| URTI/Nasopharyngitis | 7 (22%) | 5 (18%) |
| Pharyngitis/Throat infection | 6 (19%) | 1 (4%) |
| Headache | 6 (19%) | 3 (11%) |
| Arthralgia | 4 (13%) | 3 (11%) |
| Influenza/Flu | 4 (13%) | 1 (4%) |
| UTI/Pyelonephritis | 3 (9%) | 3 (11%) |
| Back pain | 1 (3%) | 3 (11%) |
| Extremity pain | 0 | 3 (11%) |

The types and incidences of adverse reactions with up to 24 months of treatment in study 3 were similar to study 1 and study 2.

In addition to those listed in Table 2, less commonly reported adverse reactions (>2%) observed in clinical trials include fatigue, pain, pharyngolaryngeal pain, pruritus, diarrhea, dizziness, nausea, bone pain, abdominal pain, erythema, flushing, edema peripheral, muscle spasms, paresthesia, dyspnea, throat irritation, asthenia, chest discomfort, infusion site pain, alanine aminotransferase increased, musculoskeletal discomfort, musculoskeletal pain, insomnia, rash, and skin irritation.

### 6.2 Immunogenicity

As with all therapeutic proteins, patients have developed IgG anti-drug antibodies (ADA) to ELELYSO. In study 1, seventeen of 32 treatment naïve patients (17/32, 53%) who were administered ELELYSO every two weeks developed ADA post-treatment (defined as ADA positive at one or more post-treatment time points). Two additional patients were antibody positive at baseline; one patient withdrew after developing an allergic reaction with the first dose of ELELYSO and the second patient had increasing antibody titers with continued treatment. In study 2, four of 28 patients (4/28, 14%) switched from imiglucerase treatment to ELELYSO treatment once every two weeks developed ADA after the switch. One additional patient who switched from imiglucerase in Study 2 was positive at baseline but did not develop increased ADA titers after ELELYSO treatment. The relevance of ADA to therapeutic response and adverse events is currently unclear.

Using neutralizing antibody assays of limited sensitivity, two treatment naïve patients (at 24 months of ELELYSO treatment) and one patient switched from imiglucerase (at 9 months of ELELYSO treatment) were determined to be positive for neutralizing activity in an *in vitro* enzyme inhibition assay and were negative in a cell based assay. For these patients there was no demonstrated association between positive neutralizing antibody assay results and therapeutic response. The significance of these findings is unknown at this time.

It is unknown if the presence of ADA to taliglucerase alfa is associated with a higher risk of infusion reactions. Patients who develop infusion or immune reactions with ELELYSO treatment should be monitored for ADA to ELELYSO. Additionally, patients with an immune reaction to other enzyme replacement therapies who are switching to ELELYSO should be monitored for ADA to ELELYSO.

Immunogenicity assay results are highly dependent on the sensitivity and specificity of the assay and may be influenced by several factors such as: assay methodology, sample handling, timing of sample collection, concomitant medication, and underlying disease. For these reasons, comparison of the incidence of antibodies to ELELYSO with the incidence of antibodies to other products may be misleading.

## 8 USE IN SPECIFIC POPULATIONS

### 8.1 Pregnancy

Category B:

Reproduction studies with taliglucerase alfa have been performed in pregnant rats at intravenous doses up to 55 mg/kg/day (about 5 times the recommended human dose of 60 Units/kg based on the body surface area) and in pregnant rabbits at intravenous doses up to 27.8 mg/kg/day (about 5 times the recommended human dose of 60 Units/kg based on the body surface area). These studies did not reveal any evidence of impaired fertility or harm to the fetus due to taliglucerase alfa. There are, however, no adequate and well controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, ELELYSO should be used during pregnancy only if clearly needed. .

### 8.3 Nursing Mothers

There are no data from studies in lactating women. It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when ELELYSO is administered to a nursing woman.

### 8.4 Pediatric Use

The safety and effectiveness of ELELYSO in pediatric patients have not been established. One 8 year-old pediatric patient experienced a serious adverse reaction (gastroenteritis).

### 8.5 Geriatric Use

During clinical studies 8 patients aged 65 or older were treated with ELELYSO. Clinical studies of ELELYSO did not include sufficient numbers of subjects aged 65 and over to determine whether they respond differently from younger subjects.

## 10 OVERDOSAGE

There is no experience with overdosage with ELELYSO.

## 11 DESCRIPTION

Taliglucerase alfa, a hydrolytic lysosomal glucocerebroside-specific enzyme for intravenous infusion, is a recombinant active form of the lysosomal enzyme, β-glucocerebrosidase, which is expressed in genetically modified carrot plant root cells cultured in a disposable bioreactor system (ProCellEx®). β-Glucocerebrosidase (β-D-glucosyl-N-acylsphingosine glucohydrolase, E.C. 3.2.1.45) is a lysosomal glycoprotein enzyme that catalyzes the hydrolysis of the glycolipid glucocerebroside to glucose and ceramide.

ELELYSO is produced by recombinant DNA technology using plant cell culture (carrot). Purified taliglucerase alfa is a monomeric glycoprotein containing 4 N-linked glycosylation sites (Mr = 60,800). Taliglucerase alfa differs from native human glucocerebrosidase by two amino acids at the N terminal and up to 7 amino acids at the C terminal. Taliglucerase alfa is a glycosylated protein with oligosaccharide chains at the glycosylation sites having terminal mannose sugars. These mannose-terminated oligosaccharide chains of taliglucerase alfa are specifically recognized by endocytic carbohydrate receptors on macrophages, the cells that accumulate lipid in Gaucher disease.

ELELYSO is supplied as a sterile, non-pyrogenic, lyophilized product. The quantitative composition of each 200 Unit vial is D-mannitol (206.7 mg), polysorbate 80 (0.56 mg), sodium citrate (30.4 mg), and taliglucerase alfa (212 Units). Citric acid may be added to adjust the pH at the time of manufacture.

A Unit is the amount of enzyme that catalyzes the hydrolysis of 1 micromole of the synthetic substrate *para*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) per minute at 37 C. After reconstitution with Sterile Water for Injection the taliglucerase alfa concentration is 40 Units/mL *[see Dosage and Administration (2)]*. Reconstituted solutions have a pH of approximately 6.0.

## 12 CLINICAL PHARMACOLOGY

### 12.1 Mechanism of Action

ELELYSO catalyzes the hydrolysis of glucocerebroside to glucose and ceramide. In clinical trials, ELELYSO reduced spleen and liver size, and improved anemia and thrombocytopenia.

### 12.3 Pharmacokinetics

In Gaucher disease patients treated with 30 or 60 units/kg (N=29), pharmacokinetics were determined with the first dose and at 38 weeks.

The pharmacokinetics of taliglucerase alfa appeared to be nonlinear with a greater than dose-proportional increase in exposure at the doses studied. The median systemic clearance (CL) values were approximately 30 L/hr and 20 L/hr for 30 and 60 units/kg, respectively. The median volume of distribution at steady state (Vss) ranged from 7.30 to 11.7 L for both dose groups. At the end of infusion, taliglucerase alfa serum concentrations fell rapidly with a median terminal half life of 18.9 to 28.7 minutes for both dose groups.

No significant accumulation or change in taliglucerase alfa pharmacokinetics over time from Weeks 1 to 38 was observed with repeated doses of 30 or 60 units/kg.

Based on the limited data, there were no significant pharmacokinetic differences between male and female patients in this study.

## 13 NONCLINICAL TOXICOLOGY

### 13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term studies in animals to evaluate carcinogenic potential or studies to evaluate mutagenic potential have not been performed with taliglucerase alfa. In a male and female fertility study in rats, taliglucerase alfa did not cause any significant adverse effect on male or female fertility parameters up to a maximum dose of 55 mg/kg/day (about 5 times the recommended human dose of 60 Units/kg based on the body surface area).

## 14 CLINICAL STUDIES

### 14.1 Study 1: Trial of ELELYSO as Initial Therapy

The safety and efficacy of ELELYSO was assessed in 31 adult patients with Type 1 Gaucher disease. The trial was a 9-month multi-center, double blind, randomized study in patients with Gaucher disease-related enlarged spleens (>8 times normal) and thrombocytopenia (<120,000 /mm3). Sixteen patients had enlarged livers and ten patients had anemia at baseline. All patients were naïve to ERT. Patients with severe neurological symptoms were excluded from the study. Patient age ranged from 19-74 years (mean age 36 years) and 48% were male. Patients were randomized to receive ELELYSO at a dose of either 30 Units/kg (n=15) or 60 Units/kg (n=16).

At baseline, mean % body weight (%BW) and multiples of normal (MN) spleen volumes were 3.1 and 3.3 (%BW) and 15.4 and 16.7 (MN) for the 30 Units/kg and 60 Units/kg dose groups, respectively. Similarly, liver volumes were 4.2 and 3.8 (%BW) and 1.7 and 1.5 (MN). Hemoglobin concentrations were 12.2 and 11.4 g/dL and platelet counts were 75,320 and 65,038/mm3, for the 30 Units/kg and 60 Units/kg dose groups, respectively. For all studies, liver and spleen volumes were measured by MRI. The changes in clinical parameters after nine months of treatment are shown in Table 3. The observed change from baseline in the primary endpoint, spleen volume, was considered to be clinically meaningful in light of the natural history of untreated Gaucher disease.

Table 3: Mean Change from Baseline to 9 months for Clinical Parameters in Treatment-Naïve Patients with Type 1 Gaucher Disease Initiating Therapy with ELELYSO

| Clinical Parameter | | 30 Units/kg (N=15) | 60 Units/kg (N=16) |
|---|---|---|---|

| | | | |
|---|---|---|---|
| Change in Spleen Volume | %BW Mean (SD)<br>MN Mean (SD) | -0.9 (0.4)<br>-4.5 (2.1) | -1.3 (1.1)<br>-6.6 (5.4) |
| | | | |
| Change in Hemoglobin g/dL | Mean (SD) | 1.6 (1.4) | 2.2 (1.4) |
| | | | |
| Change in Liver Volume | %BW Mean (SD)<br>MN Mean (SD) | -0.6 (0.5)<br>-0.2 (0.2) | -0.6 (0.4)<br>-0.3 (0.2) |
| | | | |
| Change in Platelet Count / $mm^3$ | Mean (SD) | 11,427 (20,214) | 41,494 (47,063) |
| | | | |

Twenty-six previously treatment naïve patients continued to be treated with ELELYSO in an extension of this study (Study 3) in a blinded manner for a total treatment duration of 24 months. For the respective 30 and 60 Units/kg groups, mean (±SD) spleen volume (%BW) decreased -1.4 (±0.6) and -2.0 (±2.0); hemoglobin increased 1.3 (±1.7) g/dL and 2.4 (±2.3) g/dL; liver volume (%BW) decreased -1.1 (±0.5) and -1.0 (±0.7); and platelet count increased 28,433 (±31,996) /mm3 and 72,029 (±68,157)/mm3.

**14.2 Study 2: Trial in Patients Switching from Imiglucerase to ELELYSO**

The safety and efficacy of ELELYSO was assessed in 25patients with Type 1 Gaucher disease who were switched from imiglucerase to ELELYSO. The trial was a 9-month, multi-center, open-label, single arm study in patients who had been receiving treatment with imiglucerase at doses ranging from 11 Units/kg to 60 Units/kg for a minimum of 2 years. Patients also were required to be clinically stable and to have a stable biweekly dose of imiglucerase for at least 6 months prior to enrollment. Patient age ranged from 13-66 years (mean age 45 years including pediatric) and 46% were male. Imiglucerase therapy was stopped, and treatment with ELELYSO was administered every other week at the same number of units as each patient's previous imiglucerase dose. Adjustment of dosage was allowed by study criteria if needed in order to maintain clinical parameters (i.e., hemoglobin, platelet count, spleen volume, and liver volume). One patient required a dose increase (from 9.5 Units/kg to 19 Units/kg at week 24) for a platelet count of $92,000/mm^3$ at week 22, and responded with a platelet count of $170,000mm^3$ at month 9.

Organ volumes and hematologic values remained stable on average through 9 months of ELELYSO treatment. At baseline, spleen volume %BW was 1.1% and MN was 5.5; liver volume %BW was 2.4% and MN was 1.0; mean hemoglobin was 13.6 (± 1.57) g/dL; and mean platelet count was 160,447 (± 79,086) /mm3. At the nine month endpoint, spleen volume %BW was 1.0% and MN was 5.1; liver volume %BW was 2.3% and MN was 0.9; mean hemoglobin was 13.4 (± 1.6) g/dL and mean platelet count was 165,654 (± 94,038) /mm3.

**16 HOW SUPPLIED/STORAGE AND HANDLING**

ELELYSO™ is available as a lyophilized powder, 200 Units per vial (NDC 0069 0106 01).

Store ELELYSO at 2 to 8 C (36 to 46 F). Protect vials from light.

**17 PATIENT COUNSELING INFORMATION**

- Inform patients that ELELYSO is administered under the supervision of a healthcare professional as an intravenous infusion every other week. The infusion typically takes 60 to 120 minutes.
- Advise patients that ELELYSO may cause severe allergic reactions or infusion reactions. Patients should be counseled that they should be carefully re-evaluated for treatment with ELELYSO if serious allergic reactions occur. Patients should also be counseled that infusion reactions can usually be managed by slowing the infusion rate, treatment with medications such as antihistamines, antipyretics and/or corticosteroids, and/or stopping and resuming treatment with decreased infusion rate. Patients should also be counseled that pre-treatment with antihistamines and/or corticosteroids may prevent subsequent reactions *[see Warnings and Precautions (5.1, 5.2)]*.
- Advise patients to report any adverse reactions while on ELELYSO treatment.

Manufactured and distributed by:
Pfizer Labs
Division of Pfizer Inc.
New York, NY 10017

Licensed from Protalix Biotherapeutics

LAB-0610-1.0

Elelyso™
(taliglucerase alfa) for injection

Lot

Exp

Pfizer

**Elelyso™**
(taliglucerase alfa)
for injection

**200 units/vial**

For intravenous infusion only

Distributed by
Pfizer Labs
Division of Pfizer Inc
NY, NY 10017

Licensed from
PROTALIX
Biotherapeutics

Store at 2 to 8°C (36 to 46°F).
PROTECT FROM LIGHT.
DOSAGE AND USE: See accompanying prescribing information

Each vial to be reconstituted with 5.1 ml of sterile water for injection, U.S.P to give a reconstituted volume of 5.3 ml. The extractable volume is 5 mL. Further, dilute with 0.9% sodium chloride for injection, U.S.P., for a final volume of 100 to 200 mL.

Each vial contains: taliglucerase alfa 212 units* (total amount), Mannitol 206.7 mg, Sodium Citrate 30.4 mg, Polysorbate 80 0.56 mg. Citric Acid may have been added at the time of manufacture to adjust pH.
*Extractable dose of 200 units

11707001

Pfizer

NDC 0069-0106-01

**Elelyso™**
(taliglucerase alfa)
for injection

**200 units/vial**

For intravenous infusion only

Single-use vial. Discard any unused portion.

Rx only

PRO: UPC 80%

N3 00069-10601 4

MADE IN ISRAEL

Pfizer

**Elelyso™**
(taliglucerase alfa)
for injection

**200 units/vial**

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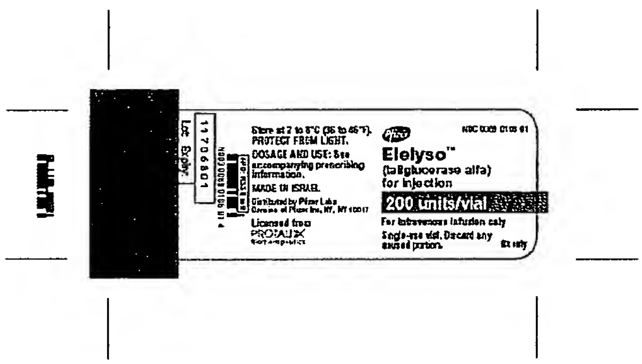


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Reference ID: 3124792

# EXHIBIT C

US005929304A

# United States Patent [19]

Radin et al.

[11] Patent Number: 5,929,304

[45] Date of Patent: Jul. 27, 1999

[54] PRODUCTION OF LYSOSOMAL ENZYMES IN PLANT-BASED EXPRESSION SYSTEMS

[75] Inventors: David N. Radin; Carole L. Cramer; Karen K. Oishi; Deborah L. Weissenborn, all of Blacksburg, Va.

[73] Assignees: Croptech Development Corporation; Virginia Tech Intellectual Properties, Inc.

[21] Appl. No.: 08/713,928

[22] Filed: Sep. 13, 1996

Related U.S. Application Data

[60] Provisional application No. 60/003,737, Sep. 14, 1995.

[51] Int. Cl.[6] ............................ C12N 5/14; C12N 15/52; C12N 15/63

[52] U.S. Cl. .......................... 800/288; 800/278; 800/287; 800/294; 800/295; 800/317.3; 435/69.1; 435/410; 435/414; 435/183; 435/206; 435/320.1; 536/23.1; 536/23.2; 536/24.1

[58] Field of Search ................................ 435/69.1, 320.1, 435/410, 414, 183, 206; 800/205, 250, 255, 278, 294, 287, 295, 288, 317.3; 536/23.2, 24.1, 23.1

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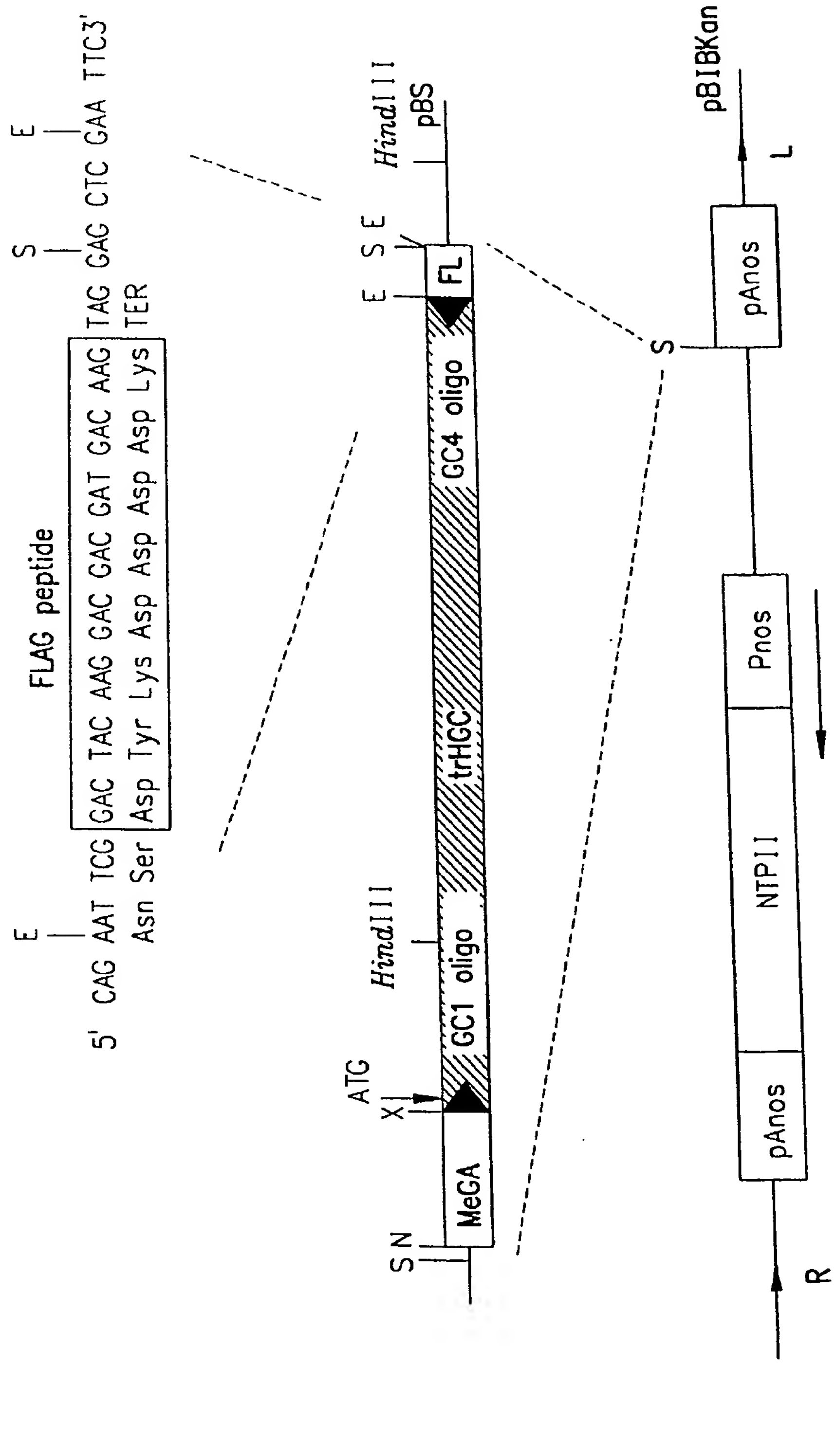
*Primary Examiner*—Elizabeth Kemmerer
*Attorney, Agent, or Firm*—Pennie & Edmonds LLP

[57] ABSTRACT

The invention relates to the production of enzymatically active recombinant human and animal lysosomal enzymes involving construction and expression of recombinant expression constructs comprising coding sequences of human or animal lysosomal enzymes in a plant expression system. The plant expression system provides for post-translational modification and processing to produce a recombinant gene product exhibiting enzymatic activity. The invention is demonstrated by working examples in which transgenic tobacco plants having recombinant expression constructs comprising human hGC and IDUA nucleotide sequences produced enzymatically active modified human glucocerebrosidase and human α-L-iduronidase. The recombinant lysosomal enzymes produced in accordance with the invention may be used for a variety of purposes, including but not limited to enzyme replacement therapy for the therapeutic treatment of human and animal lysosomal storage diseases.

73 Claims, 29 Drawing Sheets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5' CAG AAT TCG GAC TAC AAG GAC GAC GAT GAC AAG TAG GAG CTC GAA TTC3'
Asn Ser Asp Tyr Lys Asp Asp Asp Asp Lys TER
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HindIII
pBS
E S E
FL
GC4 oligo
trHGC
GC1 oligo
HindIII
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FIG.1

FIG.2A

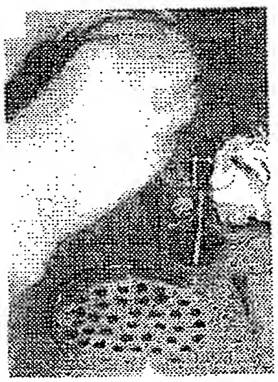


FIG.2B

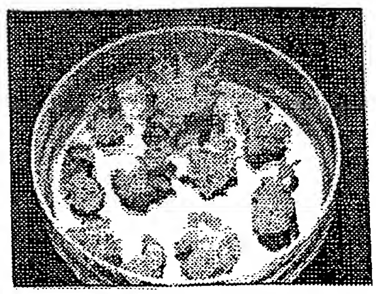


FIG.2C

FIG.2D

FIG.2E

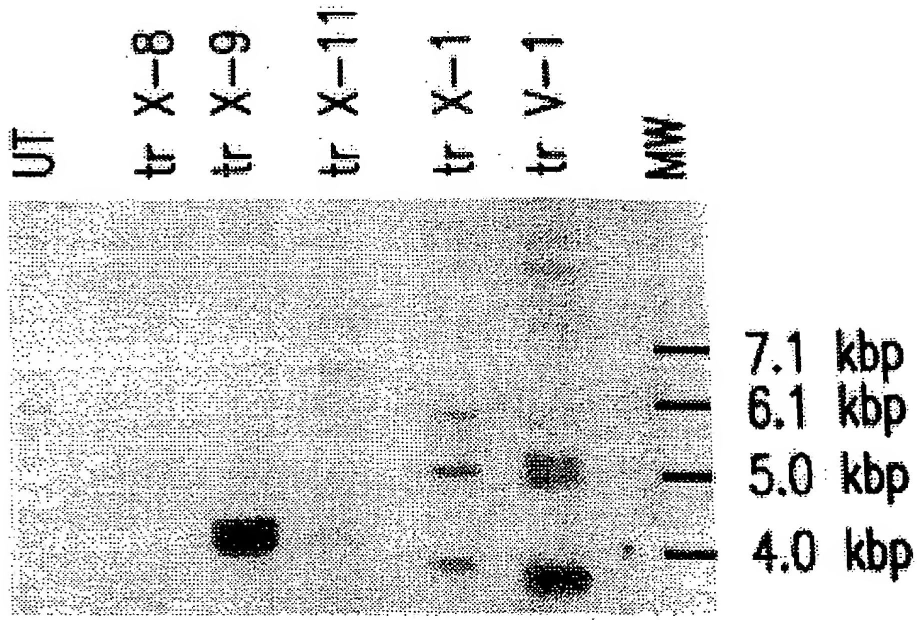



FIG.3

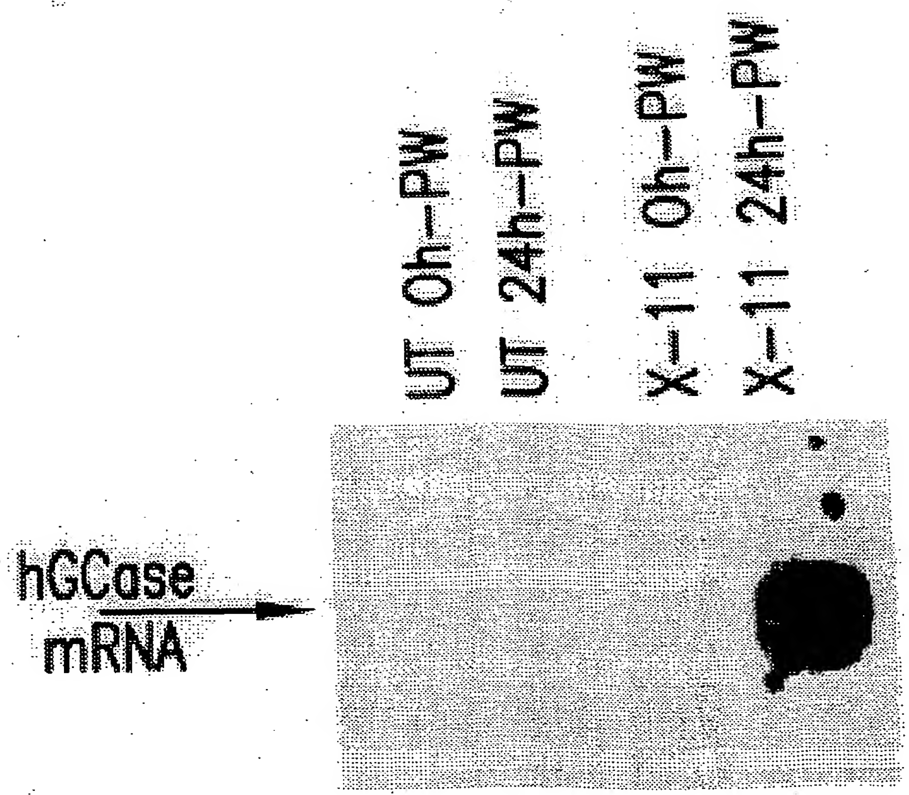



FIG.4

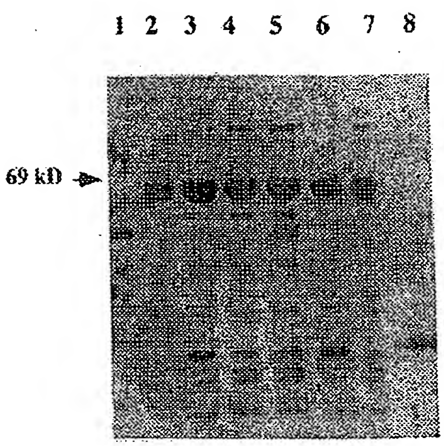



FIG.5A

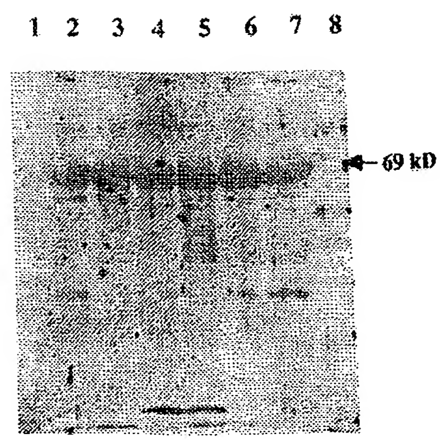



FIG.5B

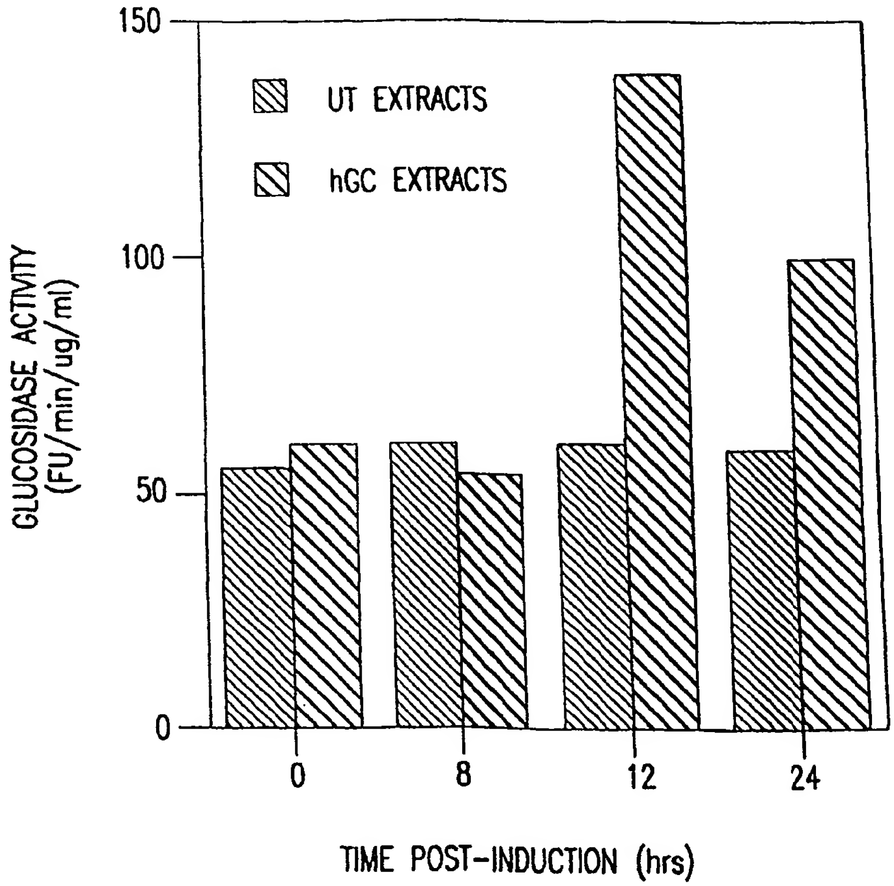

150
100
50
0
UT EXTRACTS
hGC EXTRACTS
GLUCOSIDASE ACTIVITY
(FU/min/ug/ml)
0
8
12
24
TIME POST-INDUCTION (hrs)

FIG.6

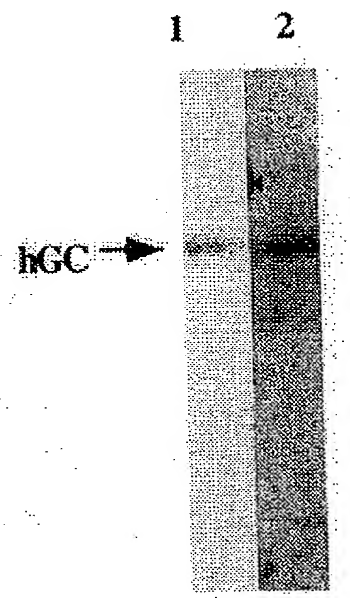



FIG.7A

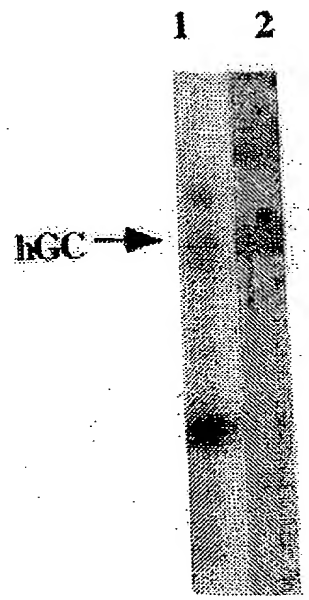



FIG.7B

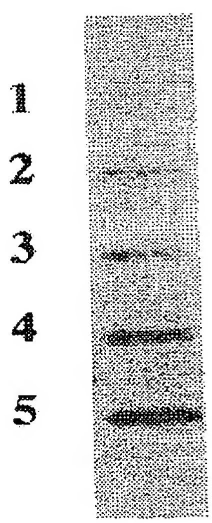



FIG.8A

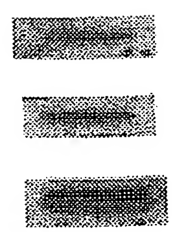


FIG.8B

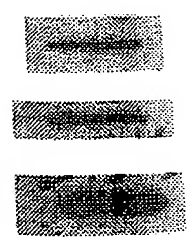


FIG.8C

FIG.9A

```
                                     123 ATGGAGTT TTCAAGTCCT TCCAGAGAGG
151  AATGTCCCAA GCCTTTGAGT AGGGTAAGCA TCATGGCTGG CAGCCTCACA
201  GGTTTGCTTC TACTTCAGGC AGTGTCGTGG GCATCAGGTG CCCGCCCCTG
251  CATCCCTAAA AGCTTCGGCT ACAGCTCGGT GGTGTGTGTC TGCAATGCCA
301  CATACTGTGA CTCCTTTGAC CCCCCGACCT TTCCTGCCCT TGGTACCTTC
351  AGCCGCTATG AGAGTACACG CAGTGGGCGA CGGATGGGGC TGAGTATGGG
401  GCCCATCCAG GCTAATCACA CGGGCACAGG CCTGCTACTG ACCCTGCAGC
451  CAGAACAGAA GTTCCAGAAA GTGAAGGGAT TTGGAGGGGC CATGACAGAT
501  GCTGCTGCTC TCAACATCCT TGCCCTGTCA CCCCCTGCCC AAAATTTGCT
551  ACTTAAATCG TACTTCTCTG AAGAAGGAAT CGGATATAAC ATCATCCGGG
601  TACCCATGGC CAGCTGTGAC TTCTCCATCC GCACCTACAC CTATGCAGAC
651  ACCCCTGATG ATTTCCAGTT GCACAACTTC AGCCTCCCAG AGGAAGATAC
701  CAAGCTCAAG ATACCCCTGA TTCACCGAGC CCTGCAGTTG GCCCAGCGTC
```

```
751   CCGTTTCACT CCTTGCCAGC CCCTGGACAT CACCCACTTG GCTCAAGACC
801   AATGGAGCGG TGAATGGGAA GGGGTCACTC AAGGGACAGC CCGGAGACAT
851   CTACCACCAG ACCTGGGCCA GATACTTTGT GAAGTTCCTG GATGCCTATG
901   CTGAGCACAA GTTACAGTTC TGGGCAGTGA CAGCTGAAAA TGAGCCTTCT
951   GCTGGGCTGT TGAGTGGATA CCCCTTCCAG TGCCTGGGCT TCACCCCTGA
1001  ACATCAGCGA GACTTCATTG CCCGTGACCT AGGTCCTACC CTCGCCAACA
1051  GTACTCACCA CAATGTCCGC CTACTCATGC TGGATGACCA ACGCTTGCTG
1101  CTGCCCCACT GGGCAAAGGT GGTACTGACA GACCCAGAAG CAGCTAAATA
1151  TGTTCATGGC ATTGCTGTAC ATTGGTACCT GGACTTTCTG GCTCCAGCCA
1201  AAGCCACCCT AGGGGAGACA CACCGCCTGT TCCCCAACAC CATGCTCTTT
1251  GCCTCAGAGG CCTGTGTGGG CTCCAAGTTC TGGGAGCAGA GTGTGCGGCT
1301  AGGCTCCTGG GATCGAGGGA TGCAGTACAG CCACAGCATC ATCACGAACC
1351  TCCTGTACCA TGTGGTCGGC TGGACCGACT GGAACCTTGC CCTGAACCCC
```

FIG.9B

```
1401  GAAGGAGGAC CCAATTGGGT GCGTAACTTT GTCGACAGTC CCATCATTGT
1451  AGACGTCACC AGGGACACGT TTTACAAACA GCCCATGTTC TACCACCTTG
1501  GCCACTTCAG CAAGTTCATT CCTGAGGGCT CCCAGAGAGT GGGGCTGGTT
1551  GCCAGTCAGA AGAACGACCT GGACGCAGTG GCACTGATGC ATCCCGATGG
1601  CTCTGCTGTT GTGGTCGTGC TAAACCGCTC CTCTAAGGAT GTGCCTCTTA
1651  CCATCAAGGA TCCTGCTGTG GGCTTCCTGG AGACAATCTC ACCTGGCTAC
1701  TCCATTCACA CCTACCTGTG GCGTCGCCAG aattcggact acaaggacga
1751  cgatgacaag tTGA
```

FIG.9C

1 50
MEFSSPSREE CPKPLSRVS IMAGSLTGLL LLQAVSWASG ARPCIPKSFG
51 100
YSSVVCVCNA TYCDSFDPP TFPALGTFSR YESTRSGRRM ELSMGPIQAN
101 150
HTGTGLLLTL QPEQKFQKV KGFGGAMTDA AALNILALSP PAQNLLLKSY
151 200
FSEEGIGYNI IRVPMASCD FSIRTYTYAD TPDDFQLHNF SLPEEDTKLK
201 250
IPLIHRALQL AQRPVSLLA SPWTSPTWLK TNGAVNGKGS LKGQPGDIYH
251 300
QTWARYFVKF LDAYAEHKL QFWAVTAENE PSAGLLSGYP FQCLGFTPEH
301 350
QRDFIARDLG PTLANSTHH NVRLLMLDDQ RLLLPHWAKV VLTDPEAAKY
351 400
VHGIAVHWYL DFLAPAKAT LGETHRLFPN TMLFASEACV GSKFWEQSVR
401 450
LGSWDRGMQY SHSIITNLL YHVVGWTDWN LALNPEGGPN WVRNFVDSPI
451 500
IVDVTKDTFY KQPMFYHLG HFSKFIPEGS QRVGLVASQK NDLDAVALMH
501 550
PDGSAVVVVL NRSSKDVPL TIKDPAVGFL ETISPGYSIH TYLWRRQnsd

ykddddk"

FIG.10

```
                                                                    60
CAATACGATA TTACCGAATA TTATACTAAA TCAAAATTTA ATTTATCATA TCGAATTATT

                                                                   120
AAACTGATAT TTCAAATTTT AATATTTAAT ATCTACTTTC AACTATTATT ACCTAATTAT

                                                                   180
CAAATGCAAA ATGTATGAGT TATTTCATAA TAGCCCGAGT TCGTATCCAA ATATTTTACA

                                                                   240
CTTGACCAGT CAACTTGACT ATATAAAACT TTACTTCAAA AAATTAAAAA AAAAAGAAAG

                                                                   300
TATATTATTG TAAAAGATAA TACTCCATTC AAAATATAAA ATGAAAAAAG TCCAGCGCGG

                                                                   360
CAACCGGGTT CCTCTATAAA TACATTTCCT ACATCTTCTC TTCTCCTCAC ATCCCATCAC

                                                                   420
TCTTCTTTTA ACAATTATAC TTGTCAATCA TCAATCCCAC AAACAACACT TTTTCTCTCC

                                                       471
TCTTTTTCCT CACCGGCGGC AGACTTACCG GTGAAATCTA GAGTAAGCAT C
```

FIG. 11

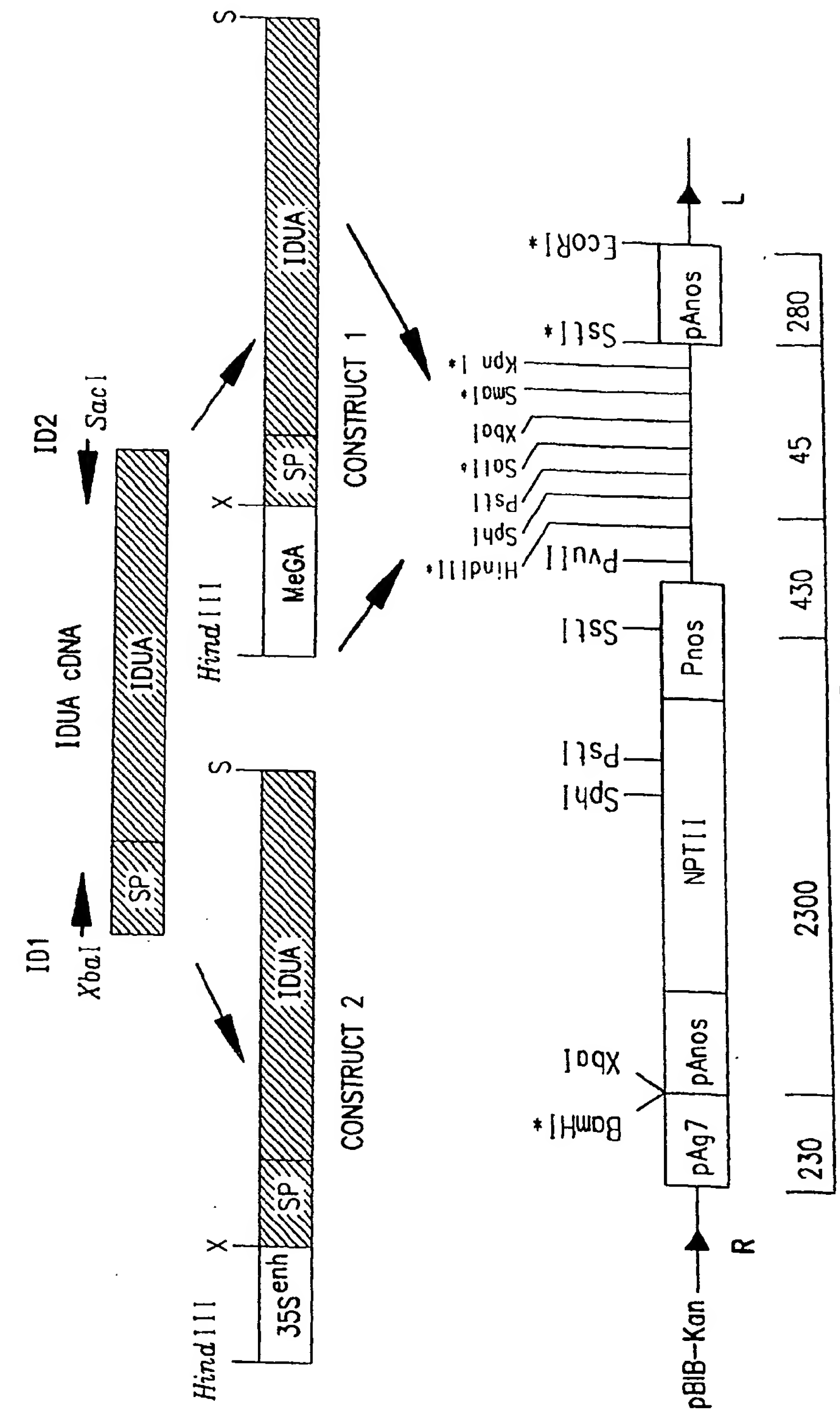
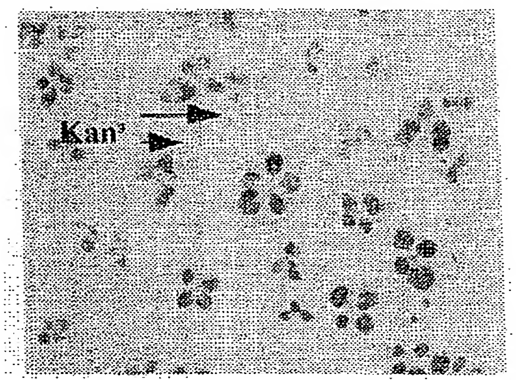

IDUA cDNA
ID1
XbaI
ID2
SacI
SP
IDUA
HindIII
X
S
MeGA
CONSTRUCT 1
35Senh
CONSTRUCT 2
EcoRI*
SstI*
Kpn I*
SmaI*
XbaI
SalI*
PstI
SphI
HindIII*
PvuII
SstI
PstI
SphI
XbaI
BamHI*
pAnos
Pnos
NPTII
pAg7
pBIB-Kan
R
L
280
45
430
2300
230

FIG.12

FIG. 13A



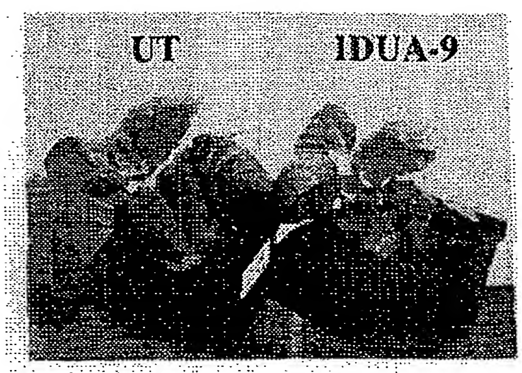




FIG. 13B

FIG. 13C

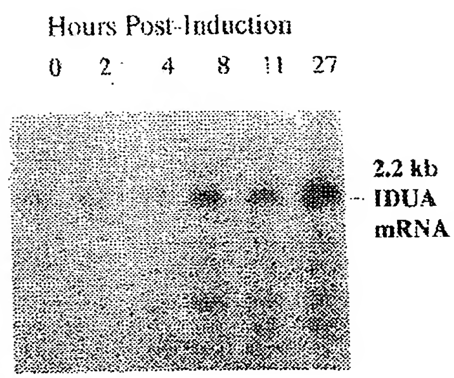



FIG.14A

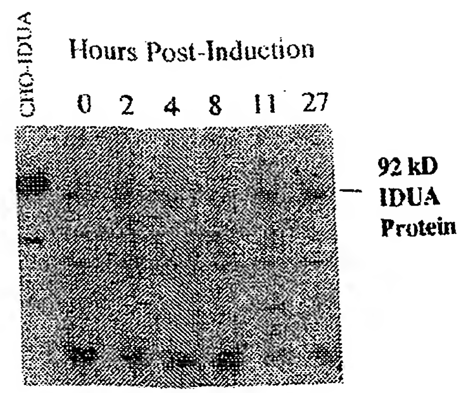



FIG.14B

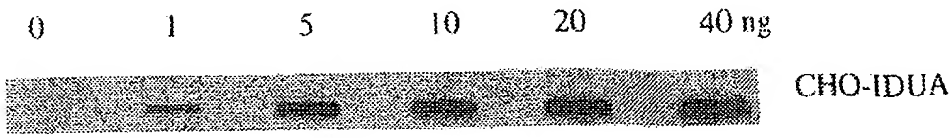



FIG. 15A

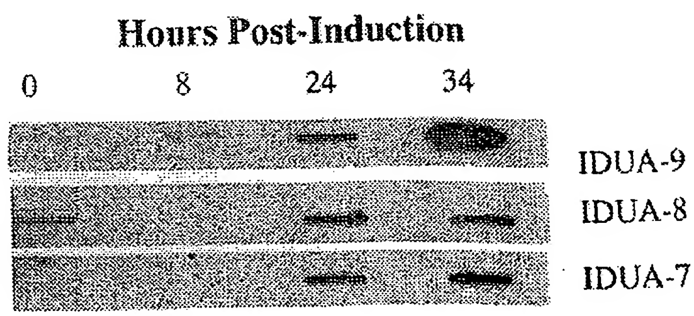



FIG. 15B

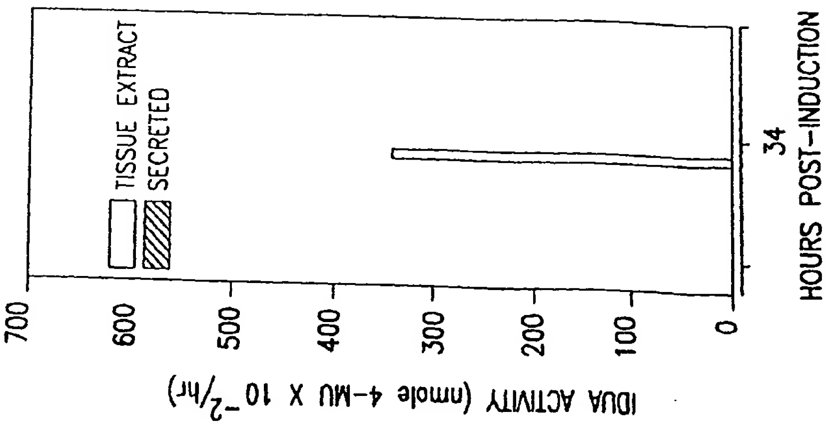



FIG.16B

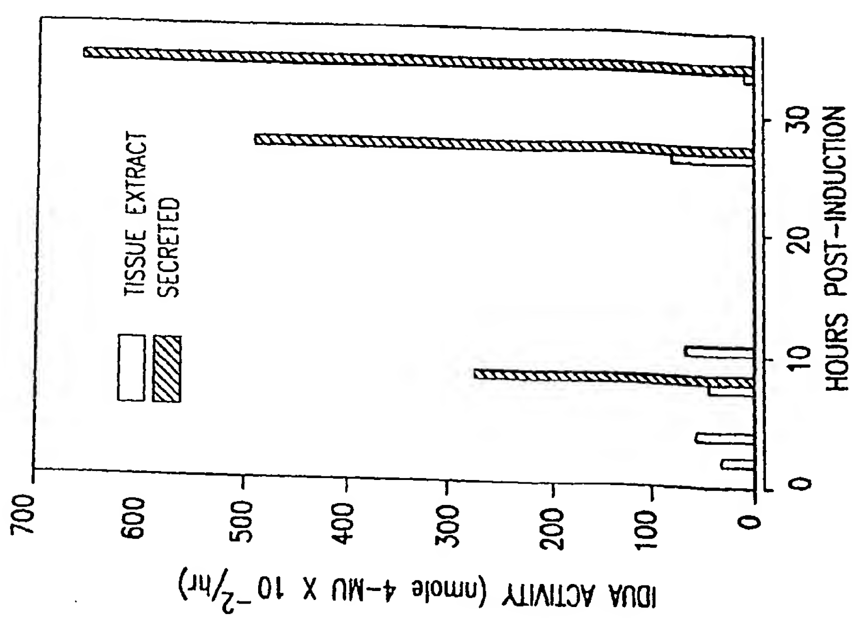



FIG.16A

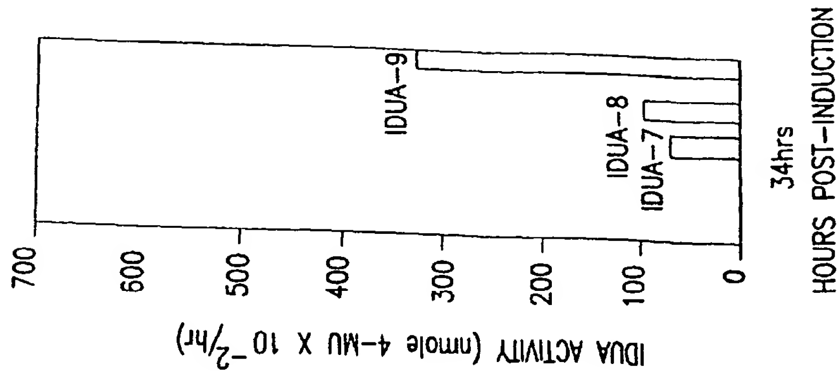



FIG.17B

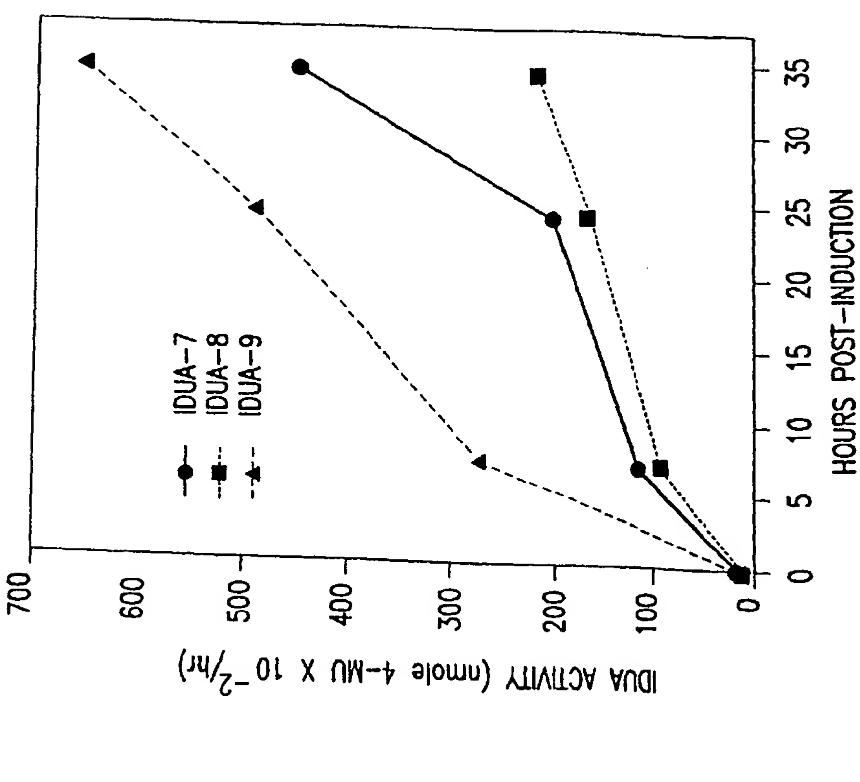



FIG.17A

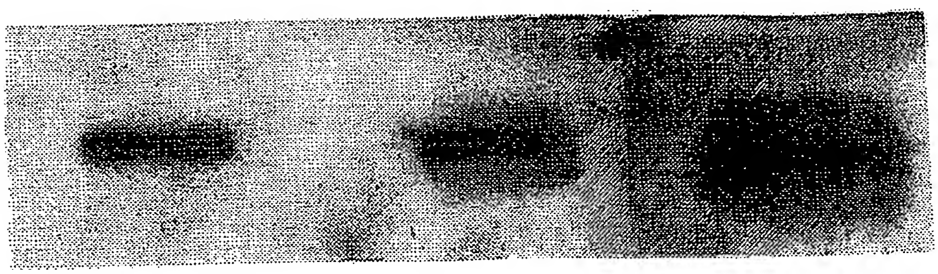



FIG. 18

```
                             90                   110
                             ATGCGTCCCCTGCGCCCCCGCGCCGCGCTGCT

      130                    150                  170
GGCGCTCCTGGCCTCGCTCCTGGCCGCGCCCCCGGTGGCCCCGGCCGAGGCCCCGCACCT

      190                    210                  230
GGTGCAGgTGGACGCGGCCCGCGCGCTGTGGCCCCTGCGGCGCTTCTGGAGGAGCACAGG

      250                    270                  290
CTTCTGCCCCCCGCTGCCACACAGCCAGGCTGACCAGTACGTCCTCAGCTGGGACCAGCA

      310                    330                  350
GCTCAACCTCGCCTATGTGGGCGCCGTCCCTCACCGCGGCATCAAGCAGGTCCGGACCCA

      370                    390                  410
CTGGCTGCTGGAGCTTGTCACCACCAGGGGGTCCACTGGACGGGGCCTGAGCTACAACTT

      430                    450                  470
CACCCACCTGGACGGGTACTTGGACCTTCTCAGGGAGAACCAGCTCCTCCCAGGGTTTGA
```

FIG. 19A

FIG.19B

```
490                 510                 530
GCTGATGGGCAGCGCCTCGGGCCACTTCACTGACTTTGAGGACAAGCAGCAGGTGTTTGA

550                 570                 590
GTGGAAGGACTTGGTCTCCAGCCTGGCCAGGAGATACATCGGTAGGTACGGACTGGCGCA

610                 630                 650
TGTTTCCAAGTGGAACTTCGAGACGTGGAATGAGCCAGACCACCACGACTTTGACAACGT

670                 690                 710
CTCCATGACCATGCAAGGCTTCCTGAACTACTACGATGCCTGCTCGGAGGGTCTGCGCGC

730                 750                 770
CGCCAGCCCCGCCCTGCGGCTGGGAGGCCCCGGCGACTCCTTCCACACCCCACCGCGATC

790                 810                 830
CCCGCTGAGCTGGGGCCTCCTGCGCCACTGCCACGACGGTACCAACTTCTTCACTGGGGA
```

FIG.19C

```
                850                 870                 890
GGCGGGCGTGCGGCTGGACTACATCTCCCTCCACAGGAAGGGTGCGCGCAGCTCCATCTC

                910                 930                 950
CATCCTGGAGCAGGAGAAGGTCGTCGCGCAcgAGATCCGGCAGCTCTTCCCCAAGTTCGC

                970                 990                1010
GGACACCCCCATTTACAACGACGAGGCGGACCCGCTGGTGGGCTGGTCCCTGCCACAGCC

               1030                1050                1070
GTGGAGGGCGGACGTGACCTACGCGGCCATGGTGGTGAAGGTCATCGCGCAGCATCAGAA

               1090                1110                1130
CCTGCTACTGGCCAACACCACCTCCGCCTTCCCCTACGCGCTCCTGAGCAACGACAATGC

               1150                1170                1190
CTTCCTGAGCTACCACCCGCACCCCTTCGCGCAGCGCACGCTCACCGCGCGCTTCCAGGT

               1210                1230                1250
CAACAACACCCGCCCGCCGCACGTGCAGCTGTTGCGCAAGCCGGTGCTCACGGCCATGGG
```

```
1270                1290                1310
GCTGCTGGCGCTGCTGGATGAGGAGCAGCTCTGGGCCGAAGTGTCGCAGGCCGGGACCGT

1330                1350                1370
CCTGGACAGCAACCACACGGTGGGCGTCCTGGCCAGCGCCCACCGCCCCCAGGGCCCGGC

1390                1410                1430
CGACGCCTGGCGCGCCGCGGTGCTGATCTACGCGAGCGACGACACCCGCGCCCACCCCAA

1450                1470                1490
CCGCAGCGTCGCGGTGACCCTGCGGCTGCGCGGGGTGCCCCCCGGCCCGGGCCTGGTCTA

1510                1530                1550
CGTCACGCGCTACCTGGACAACGGGCTCTGCAGCCCCGACGGCGAGTGGCGGCGCCTGGG

1570                1590                1610
CCGGCCCGTCTTCCCCACGGCAGAGCAGTTCCGGCGCATGCGCGCGGCTGAGGACCCGGT
```

FIG.19D

FIG.19E

```
    1630                1650                1670
GGCCGCGGCGCCCCGCCCCTTACCCGCCGGCGGCCGCCTGACCCTGCGCCCCGCGCTGCG

    1690                1710                1730
GCTGCCGTCGCTTTTGCTGGTGCACGTGTGTGCGCGCCCCGAGAAGCCGCCCGGGCAGGT

    1750                1770                1790
CACGCGGCTCCGCGCCCTGCCCCTGACCCAAGGGCAGCTGGTTCTGGTCTGGTCGGATGA

    1810                1830                1850
ACACGTGGGCTCCAAGTGCCTGTGGACATACGAGATCCAGTTCTCTCAGGACGGTAAGGC

    1870                1890                1910
GTACACCCCGGTCAGCAGGAAGCCATCGACCTTCAACCTCTTTGTGTTCAGCCCAGACAC

    1930                1950                1970
AGGTGCTGTCTCTGGCTCCTACCGAGTTCGAGCCCTGGACTACTGGGCCCGACCAGGCCC

    1990                2010                2030
CTTCTCGGACCCTGTGCCGTACCTGGAGGTCCCTGTGCCAAGAGGGCCCCCATCCCCGGG
```

```
        2050                 2070                 2090
CAATCCATGAGCCTGTGCTGAGCCCCAGTGGGTTGCACCTCCACCGGCAGTCAGCGAGCT

        2110                 2130                 2150
GGGGCTGCACTGTGCCCATGCTGCCCTCCCATCACCCCCTTTGCAATATATTTTT
```

FIG. 19F

FIG.20A

```
          10                          30                          50
MRPLRPRAALLALLASLLAAPPVAPAEAPHLVHVDAARALWPLRRFWRSTGFCPPLPHSQ

          70                          90                         110
ADQYVLSWDQQLNLAYVGAVPHRGIKQVRTHWLLELVTTRGSTGRGLSYNFTHLDGTLDL

         130                         150                         170
LRENQLLPGFELMGSASGHFTDFEDKQQVFEWKDLVSSLARRYIGRYGLAHVSKWNFETW

         190                         210                         230
NEPDHHDFDNVSMTMQGFLNYYDACSEGLRAASPALRLGGPGDSFHTPPRSPLSWGLLRH

         250                         270                         290
CHDGTNFFTGEAGVRLDYISLHRKGARSSISILEQEKVVAQEIRQLFPKFADTPIYNDEA

         310                         330                         350
DPLVGWSLPQPWRADVTYAAMVVKVIAQHQNLLLANTTSAFPYALLSNDNAFLSYHPHPF

         370                         390                         410
AQRTLTARFQVNNTRPPHVQLLRKPVLTAMGLLALLDEEQLWAEVSQAGTVLDSNHTVGV
```

```
430                 450                 470
LASAHRPQGPADAWRAAVLIYASDDTRAHPNRSVAVTLRLRGVPPGPGLVYVTRYLDNGL

490                 510                 530
CSPDGEWRRLGRPVFPTAEQFRRMRAAEDPVAAAPRPLPAGGRLTLRPALRLPSLLLVHV

550                 570                 590
CARPEKPPGQVTRLRALPLTQGQLVLVWSDEHVGSKCLWTYEIQFSQDGKAYTPVSRKPS

610                 630                 650
TFNLFVFSPDTGAVSGSYRVRALDYWARPGPFSDPVPYLEVPVPRGPPSPGNP
```

FIG.20B

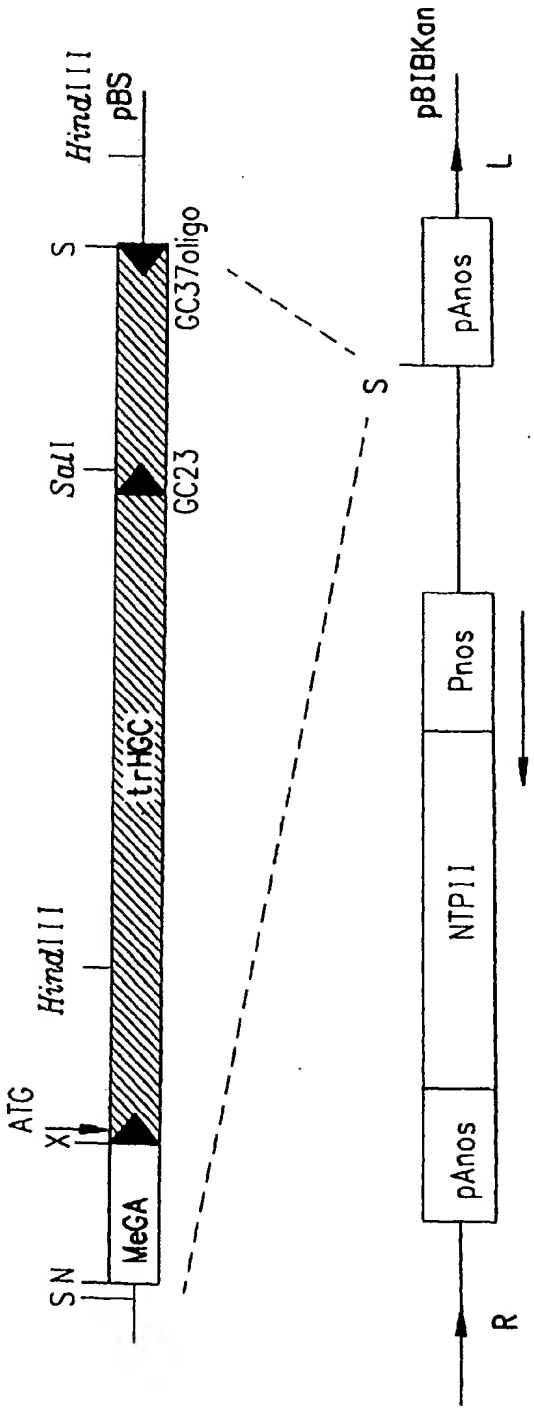

HindIII
pBS
S
GC37oligo
SalI
GC23
trHGC
HindIII
ATG
XI
MeGA
S N
pBIBKan
L
pAnos
S
Pnos
NTPII
pAnos
R

FIG.21

# PRODUCTION OF LYSOSOMAL ENZYMES IN PLANT-BASED EXPRESSION SYSTEMS

This application is a continuation-in-part of provisional application Ser. No. 60/003,737, filed Sept. 14, 1995, the disclosure of which is incorporated herein in its entirety.

This invention was made with United States government support under grant nos. NS32369 and DK48570 awarded by the National Institutes of Health. The government has certain rights in the invention.

TABLE OF CONTENTS



## 1. FIELD OF THE INVENTION

The present invention relates to the production of human and animal lysosomal enzymes in plants comprising expressing the genetic coding sequence of a human or animal lysosomal enzyme in a plant expression system. The plant expression system provides for post-translational modification and processing to produce recombinant protein having enzymatic activity.

The invention is demonstrated herein by working examples in which transgenic tobacco plants produce a modified human glucocerebrosidase (hGC) and a human α-L-iduronidase (IDUA), both of which are enzymatically active.

The recombinant lysosomal enzymes produced in accordance with the invention may be used for a variety of purposes including but not limited to enzyme replacement therapy for the therapeutic treatment of lysosomal storage diseases, research for development of new approaches to medical treatment of lysosomal storage diseases, and industrial processes involving enzymatic substrate hydrolysis.

## 2. BACKGROUND OF THE INVENTION

### 2.1. LYSOSOMAL STORAGE DISEASES

Lysosomes, which are present in all animal cells, are acidic cytoplasmic organelles that contain an assortment of hydrolytic enzymes. These enzymes function in the degradation of internalized and endogenous macromolecular substrates. When there is a lysosomal enzyme deficiency, the deficient enzyme's undegraded substrates gradually accumulate within the lysosomes causing a progressive increase in the size and number of these organelles within the cell. This accumulation within the cell eventually leads to malfunction of the organ and to the gross pathology of a lysosomal storage disease, with the particular disease depending on the particular enzyme deficiency. More than thirty distinct, inherited lysosomal storage diseases have been characterized in humans.

A few examples of lysosomal storage diseases (and their associated deficient enzymes) include Fabry disease (α-galactosidase), Farber disease (ceramidase), Gaucher disease (glucocerebrosidase), $G_{m1}$ gangliosidosis (β-galactosidase), Tay-Sachs disease (β-hexosaminidase), Niemann-Pick disease (sphingomyelinase), Schindler disease (α-N-acetylgalactosaminidase), Hunter syndrome (iduronate-2-sulfatase), Sly syndrome (β-glucuronidase), Hurler and Hurler/Scheie syndromes (iduronidase), and I-Cell/San Filipo syndrome (mannose 6-phosphate transporter).

One proven treatment for lysosomal storage diseases is enzyme replacement therapy in which an active form of the enzyme is administered directly to the patient. However, abundant, inexpensive and safe supplies of therapeutic lysosomal enzymes are not commercially available for the treatment of any of the lysosomal storage diseases.

#### 2.1.1. GAUCHER DISEASE AND TREATMENT

Gaucher disease is the most common lysosomal storage disease in humans, with the highest frequency encountered in the Ashkenazi Jewish population. About 5,000 to 10,000 people in the United States are afflicted with this disease (Grabowski, 1993, Adv. Hum. Genet. 21:377–441). Gaucher disease results from a deficiency in glucocerebrosidase (hGC; glucosylceramidase; acid β-glucosidase; EC 3.2.1.45). This deficiency leads to an accumulation of the enzyme's substrate, glucocerebroside, in reticuloendothelial cells of the bone marrow, spleen and liver, resulting in significant skeletal complications such as bone marrow expansion and bone deterioration, and also hypersplenism, hepatomegaly, thrombocytopenia, anemia and lung complications (Grabowski, 1993, supra; Lee, 1982, Prog. Clin. Biol. Res. 95:177–217; Brady et al., 1965, Biochem. Biophys. Res. Comm. 18:221–225).

hGC replacement therapy has revolutionized the medical care and management of Gaucher disease, leading to significant improvement in the quality of life of many Gaucher patients (Pastores et al., 1993, Blood 82:408–416; Fallet et al., 1992, Pediatr. Res. 31:496–502). Studies have shown that regular, intravenous administration of specifically modified hGC (Ceredase™, Genzyme Corp.) can result in dramatic improvements and even reversals in the hepatic, splenic and hematologic manifestations of the disease (Pastores et al., 1993, supra; Fallet et al., 1992, supra; Figueroa et al., 1992, N. Eng. J. Med 327:1632–1636; Barton et al., 1991, N. Eng. J. Med. 324:1464–1470; Beutler et al., 1991, Blood 78:1183–1189). Improvements in associated skeletal and lung complications are possible, but require larger doses of enzyme over longer periods of time.

Despite the benefits of hGC replacement therapy, the source and high cost of the enzyme seriously restricts its availability. Until recently, the only commercial source of purified hGC has been from pooled human placentae, where ten to twenty kilograms (kg) of placentae yield only 1 milligram (mg) of enzyme. From five hundred to two thousand kilograms of placenta (equivalent to 2,000–8,000 placentae) are required to treat each patient every two weeks. Current costs for HGC replacement therapy range from \$55 to \$220/kg patient body weight every two weeks, or from \$70,000 to \$300,000/year for a 50 kg patient. Since the need for therapy essentially lasts for the duration of a patient's life, costs for the enzyme alone may exceed \$15,000,000 during 30 to 70 years of therapy.

A second major problem associated with treating Gaucher patients with glucocerebrosidase isolated from human tissue (and perhaps even from other animal tissues) is the risk of exposing patients to infectious agents which may be present in the pooled placentae, e.g., human immuno-deficiency virus (HIV), hepatitis viruses, and others.

Accordingly, a new source of hGC is needed to effectively reduce the cost of treatment and to eliminate the risk of exposing Gaucher patients to infectious agents.

#### 2.1.2. HURLER SYNDROME AND TREATMENT

Hurler syndrome is the most common of the group of human lysosomal storage disorders known as the mucopolysaccharidoses (MPS) involving an inability to degrade dermatan sulfate and heparan sulfate. Hurler patients are deficient in the lysosomal enzyme, α-L-iduronidase (IDUA), and the resulting accumulation of glucosaminoglycans in the lysosomes of affected cells leads to a variety of clinical manifestations (Neufeld & Ashwell, 1980, *The Biochemistry of Glycoproteins and Proteoglycans*, ed. W. J. Lennarz, Plenum Press, N.Y.; pp. 241–266) including developmental delay, enlargement of the liver and spleen, skeletal abnormalities, mental retardation, coarsened facial features, corneal clouding, and respiratory and cardiovascular involvement. Hurler/Scheie syndrome (MPS I H/S) and Scheie syndrome (MPS IS) represent less severe forms of the disorder but also involve deficiencies in IDUA. Molecular studies on the genes and cDNAs of MPS I patients has led to an emerging understanding of genotype and clinical phenotype (Scott et al., 1990, Am. J. Hum. Genet. 47:802–807). In addition, both a canine and feline form of MPS I have been characterized (Haskins et al., 1979, Pediat. Res. 13:1294–1297; Haskins and Kakkis, 1995, Am. J. Hum. Genet. 57:A39 Abstr. 194; Shull et al., 1994, Proc. Natl. Acad. Sci. USA, 91:12937–12941) providing an effective in vivo model for testing therapeutic approaches.

The efficacy of enzyme replacement in the canine model of Hurler syndrome using human IDUA generated in CHO cells was recently reported (Kakkis et al., 1995, Am. J. Hum. Genet. 57:A39 (Abstr.); Shull et al., 1994, supra). Weekly doses of approximately 1 mg administered over a period of 3 months resulted in normal levels of the enzyme in liver and spleen, lower but significant levels in kidney and Lungs and very low levels in brain, heart, cartilage and cornea (Shull et al., 1994, supra. Tissue examinations showed normalization of lysosomal storage in the liver, spleen and kidney, but no improvement in heart, brain and corneal tissues. One dog was maintained on treatment for 13 months and was clearly more active with improvement in skeletal deformities, joint stiffness, corneal clouding and weight gain (Kakkis et al., 1995, supra. A single higher-dose experiment was quite promising and showed detectable IDUA activity in the brain and cartilage in addition to tissues which previously showed activity at the lower does. Additional higher-dose experiments and trials involving longer administration are currently limited by availability of recombinant enzyme. These

experiments underscore the potential of replacement therapy for Hurler patients and the severe constraints on both canine and human trials due to limitations in recombinant enzyme production using current technologies.

2.2. BIOSYNTHESIS OF LYSOSOMAL ENZYMES

Soluble lysosomal enzymes share initial steps of biosynthesis with secretory proteins, i.e., synthesis on the ribosome, binding of the N-terminal signal peptide to the surface of the rough endoplasmic reticulum (ER), transport into the lumen of the ER where the signal peptide is cleaved, and addition of oligosaccharides to specific asparagine residues (N-linked), followed by further modifications of the nascent protein in the Golgi apparatus (von Figura and Hasilik, 1986, Annu. Rev. Biochem. 55:167–193). The N-linked oligosaccharides can be complex, diverse and heterogeneous, and may contain high-mannose residues. The proteins undergo further processing in a post-ER, pre-Golgi compartment and in the cis-Golgi to form either an N-linked mannose 6-phosphate (M-6-P) oligosaccharide-dependent or N-linked M-6-P oligosaccharide-independent recognition signal for lysosomal localized enzymes (Kornfeld & Mellman, 1989, Ann. Rev. Cell Biol., 5:483–525; Kaplan et al., 1977, Proc. Natl. Acad. Sci. USA 74:2026). The presence of the M-6-P recognition signal results in the binding of the enzyme to M-6-P receptors (MPR). These bound enzymes remain in the cell, are eventually packaged into lysosomes, and are thus segregated from proteins targeted for secretion or to the plasma membrane.

Although many lysosomal enzymes are soluble and are transported to lysosomes by MPRs, integral membrane and membrane-associated proteins (notably hGC) are targeted and transported to lysosomes independent of the M-6-P/MPR system (Kornfeld & Mellman, 1989, Erickson et al., 1985). hGC does not become soluble after translation, but instead becomes associated with the lysosomal membrane by means which have not been elucidated (von Figura & Hasilik, 1986, Annu. Rev. Biochem. 55:167–193; Kornfeld and Mellman, 1989, Annu. Rev. Cell Biol. 5:483–525).

hGC is synthesized as a single polypeptide (58 kDa) with a signal sequence (2 kDa) at the amino terminus. The signal sequence is co-translationally cleaved and the enzyme is glycosylated with a heterogeneous group of both complex and high-mannose oligosaccharides to form a precursor. The glycans are predominately involved in protein conformation. The "high mannose" precursor, which has a molecular weight of 63 Kda, is post-translationally processed in the Golgi to a 66 Kda intermediate, which is then further modified in the lysosome to the mature enzyme having a molecular weight of 59 Kda (Jonsson et al., 1987, Eur. J. Biochem. 164:171; Erickson et al., 1985, J. Biol. Chem., 260:14319).

The mature hGC polypeptide is composed of 497 amino acids and contains five N-glycosylation amino acid consensus sequences (Asn-X-Ser/Thr). Four of these sites are normally glycosylated. Glycosylation of the first site is essential for the production of active protein. Both high-mannose and complex oligosaccharide chains have been identified (Berg-Fussman et al., 1993, J. Biol. Chem. 268:14861–14866). hGC from placenta contains 7% carbohydrate, 20% of which is of the high-mannose type (Grace & Grabowski, 1990, Biochem. Biophys. Res. Comm. 168:771–777). Treatment of placental hGC with neuraminidase (yielding an asialo enzyme) results in increased clearance and uptake rates by rat liver cells with a concomitant increase in hepatic enzymatic activity (Furbish et al., 1981, Biochim. Biophys. Acta 673:425–434). This glycan-modified placental hGC is currently used as a therapeutic agent in the treatment of Gaucher's disease. Biochemical and site-directed mutagenesis studies have provided an initial map of regions and residues important to folding, activator interaction, and active site location (Grace et al., 1994, J. Biol. Chem. 269:2283–2291).

The complete complementary DNA (cDNA) sequence for hGC has been published (Tsuji et al., 1986, J. Biol. Chem. 261:50–53; Sorge et al., 1985, Proc. Natl. Acad. Sci. USA 82:7289–7293), and *E. coil* containing the hGC cDNA sequence cloned from fibroblast cells, as described (Sorge et al., 1985, supra), is available from the American Type Culture Collection (ATCC) (Accession No. 65696).

Recombinant methodologies have the potential to provide a safer and less expensive source of lysosomal enzymes for replacement therapy. However, production of active enzymes, e.g., hGC, in a heterologous system requires correct targeting to the ER, and appropriate N-linked glycosylation at levels or efficiencies that avoid ER-based degradation or aggregation. Since mature lysosomal enzymes must be glycosylated to be active, bacterial systems cannot be used. For example, hGC expressed in *E. coli* is enzymatically inactive (Grace & Grabowski, 1990, supra).

Active monomers of hGC have been purified from insect cells (Sf9 cells) and Chinese hamster ovary (CHO) cells infected or transfected, respectively, with hGC cDNA (Grace & Grabowski, 1990, supra; Grabowski et al., 1989, Enzyme 41:131–142). A method for producing recombinant hGC in CHO cell cultures and in insect cell cultures was recently disclosed in U.S. Pat. No. 5,236,838. Recombinant hGC produced in these heterologous systems had an apparent molecular weight ranging from 64 to 73 kDa and contained from 5 to 15% carbohydrate (Grace & Grabowski, 1990, supra; Grace et al., 1990, J. Biol. Chem. 265:6827–6835). These recombinant hGCs had kinetic properties identical to the natural enzyme isolated from human placentae, as based on analyses using a series of substrate and transition state analogues, negatively-charged lipid activators, protein activators (saposin C), and mechanism-based covalent inhibitors (Grace et al., 1994, supra; Berg-Fussman et al., 1993, supra; Grace et al., 1990, J. Biol. Chem. 265:6827–6835; Grabowski et al., 1989, supra). However, both insect cells and CHO cells retained most of the enzyme rather than secreting it into the medium, significantly increasing the difficulty and cost of harvesting the pure enzyme (Grabowski et al., 1989, supra).

Accordingly, a recombinant system is needed that can produce human or animal lysosomal enzymes in an active form at lower cost, and that will be appropriately targeted for ease of recovery.

2.3. MAMMALIAN LYSOSOMES VERSUS PLANT VACUOLES

Because plants are eukaryotes, plant expression systems have advantages over prokaryotic expression systems, particularly with respect to correct processing of eukaryotic gene products. However, unlike animal cells, plant cells do not possess lysosomes. Although the plant vacuole appears functionally analogous to the lysosome, plants do not contain MPRs (Chrispeels, 1991, Ann. Rev. Pl. Phys. Pl. Mol. Biol. 42:21–53; Chrispeels and Tague, 1991, Intl. Rev. Cytol. 125:1–45), and the mechanisms of vacuolar targeting can differ significantly from those of lysosomal targeting. For example, the predominant mechanism of vacuolar targeting in plants does not appear to be glycan-dependent, but appears to be based instead on C- or N-terminal peptide sequences (Gomez & Chrispeels, 1993, Plant Cell 5:1113–1124; Chrispeels & Raikhal, 1992, Cell 68:613–618;

Holwerda et al., 1992, Plant Cell 4:307–318; Neuhaus et al., 1991, Proc. Natl. Acad. Sci. USA 88:10362–10366; Chrispeels, 1991, supra; Chrispeels & Tague, 1991, supra; Holwerda et al., 1990, Plant Cell 2:1091–1106; Voelker et al., 1989, Plant Cell 1:95–104). As a result, plants have not been viewed as appropriate expression systems for lysosomal enzymes which must be appropriately processed to produce an active product.

## 3. SUMMARY OF THE INVENTION

The present invention relates to the production of human or animal lysosomal enzymes in transformed or transfected plants, plant cells or plant tissues, and involves constructing and expressing recombinant expression constructs comprising lysosomal enzyme coding sequences in a plant expression system. The plant expression system provides appropriate co-translational and post-translational modifications of the nascent peptide required for processing, e.g., signal sequence cleavage, glycosylation, and sorting of the expression product so that an enzymatically active protein is produced. Using the methods described herein, recombinant lysosomal enzymes are produced in plant expression systems from which the recombinant lysosomal enzymes can be isolated and used for a variety of purposes. The present invention is exemplified by the genetic-engineering of transgenic tobacco plants with three lysosomal enzyme expression constructs. One construct comprises a nucleotide sequence encoding a modified human glucocerebrosidase (hGC), specifically a hGC fused at its C-terminal to the eight amino acid FLAG™ peptide (hGC:FLAG™). Another construct comprises nucleotide sequence encoding a human α-L-iduronidase (IDUA). The third construct zomprises a nucleotide sequence encoding a human glucocerebrosidase (hGC). Transgenic tobacco plants having the expression constructs produce lysosomal enzymes that are enzymatically active.

The plant expression systems and the recombinant lysosomal enzymes produced therewith have a varieta of uses, including but not limited to: (1) the production of enzymatically active lysosomal enzymes for the treatment of lysosomal storage diseases; (2) the production of altered or mutated proteins, enzymatically active or otherwise, to serve as precursors or substrates for further in vivo or in vitro processing to a specialized industrial form for research or therapeutic uses, such as to produce a more effective therapeutic enzyme; (3) the production of antibodies against lysosomal enzymes for medical diagnostic use; and (4) use in any commercial process that involves substrate hydrolysis.

## 4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. hGC:FLAG™ cDNA plant expression construct and transformation vector. The MeGA:hGC:FLAG™ construct in a pBS intermediate vector is excised and inserted into the SstI site of the binary plant transformation vector pBIB-KAN to form plasmid CTPro1:hGC:FLAG. R and L represent T-DNA right and left borders, respectively, which precisely delineate the DNA inserted into the plant genome. NPTII=kanamycin selectable marker, FL=FLAG™ epitope (the nucleotide and amino acid sequences shown are SEQ ID NOS:13 & 14, respectively), pAnos=polyadenylation/terminator signal, Pnos=promoter sequence from *Agrobacterium tumefaciens* nopaline synthetase gene. PCR-amplification primers for hGC were: GC1 (5'TTG tcTAGaGTAAGCATCATGGCTGGC3') (SEQ ID NO:1); and GC4 (5'cac gaattCTGGCGACGCCACAGGTAGGTGTGA3') (SEQ ID NO:2); hGC-derived sequences are in upper case; restriction sites are underlined. Restriction enzymes: E, EcoRI; S, SstI; N, NotI; X, XbaI.

FIGS. 2A–E. Transformation and generation of tobacco plants carrying the MeGA:hGC:FLAG™ construct. FIG. 2A. Agrobacterium-mediated transformation of tobacco leaf discs. Leaf discs were inoculated with a cell suspension of *A. tumefaciens* strains carrying the plasmid CTPro1:hGC:FLAG. FIG. 2B. Development of shoots on selection media 22 days post-inoculation. FIG. 2C. Development of roots on rooting media 27 days post-inoculation. Use of rooting media containing kanamycin clearly differentiated between transgenic shoots which formed roots and "false positive" shoots which did not form roots on selective media. FIG. 2D. Transformed plants three weeks after transfer to soil. FIG. 2E. Transformed plant 10 weeks after transfer to soil.

FIG. 3. Genomic Southern hybridization analysis of control and transgenic plants. Total genomic DNA was isolated from an untransformed control plant (UT) and independent transformants generated from *Nicotiana tabacum* cv. Xanthi (X-1, X-8, X-9, X-11) and cv. VA116 (V1). Five to 10 μg of total genomic DNA were digested with HindIII and resolved on a TBE agarose gel. The DNA was blotted to nitrocellulose membrane and probed with a $^{32}$P-labeled hGC:FLAG™ sequence from a gel-purified 1.7 kb HindIII fragment isolated from the pBS intermediate vector containing the MeGA:hGC:FLAG™ expression construct (see FIG. 1).

FIG. 4. Induction of hGC:FLAG™ mRNA levels in transgenic plants. Total RNA was isolated by standard guanidino-thiocyanate methods from UT and X-11 leaf tissue at 0 and 24 hr post-mechanical gene activation (MGA). Five μg of total RNA was glyoxylated, size-separated on a 1.2% agarose gel, transferred to NitroPure (MSI) filters and probed with a $^{32}$P-labeled hGC:FLAG™ gene sequence from a gel-purified 1.7 kb HindIII fragment isolated from the pBS intermediate vector shown in FIG. 1.

FIGS. 5A–B. Induction of hGC:FLAG™ fusion protein in transgenic tobacco plants as detected by Western analysis using anti-FLAG™ antibodies and anti-hGC antibodies. Leaf tissue from X-11 was induced by MGA at time 0 at room temperature, harvested at 2, 4, 8, 16, and 24 hrs, and frozen at −20° C. prior to extraction. hGC:FLAG™ was solubilized by grinding the tissue in a coffee bean grinder with dry ice and homogenized in 1% Triton X-100, 1% taurocholate, 25 mM sodium citrate pH 7.0, 4 mM β-mercaptoethanol, and 5 mM ethylenediaminetetraacetic acid (EDTA), followed by two cycles of freezing and thawing of the homogenate. Both protein concentration and enzyme activity of cell free extracts were determined. FIG. 5A. Ten μg of total soluble protein were analyzed by Western immunoblot using anti-FLAG™ antibodies. Lane 1, 24 ng of FLAG™-tagged control protein; lane 2, X-11 at time 0; lane 3, X-11 at 2 hr; lane 4, X-11 at 4 hrs; lane 5, X-11 at 8 hrs; lane 6, X-11 at 12 hrs; lane 7, X-11 at 24 hrs; lane 8, UT (control plant) at 12 hrs. FIG. 5B. Forty μg of total soluble protein were analyzed by Western immunoblot using anti-hGC antibodies. Lane 1, UT at time 0; lane 2, X-11 at time 0; lane 3, X-11 at 2 hrs; lane 4, X-11 at 4 hrs; lane 5, X-11 at 8 hrs; lane 6, X-11 at 12 hrs, lane 7, X-11 at 24 hrs; lane 8, UT at 8 hrs. The maximum level of hGC:FLAG™ expression was found between 8–12 hrs post-MGA.

FIG. 6. Total β-glucosidase (endogenous plant β-glucosidase and hGC) activity post-MGA of X-11 leaf tissue. One-tenth μg of cell free extract was assayed for

ability to convert the fluorometric substrate, 4-methylumbelliferyl-D-glucopyranoside (4MuGlc) to 4MU at 37° C., as measured in a fluorometer (Hoefer DyNA Quant-200, Hoefer, Pharmacia, Biotech. Inc.) with excitation at 365 nm and emission at 460 nm. FU=fluorometer units; Time=hrs post-inducti(on (i.e., wounding of tissue or MGA).

FIGS. 7A–B. Affinity purification of hGC:FLAG™ fusion protein. FIG. 7A. Commassie blue stained SDS-PAGE gel and Western analysis of FLAG™ affinity-purified hGC-:FLAG™. Lane 1, Coomassie blue stained SDS-PAGE gel of 0.1 μg FLAG™ affinity-purified hGC:FLAG™; Lane 2, Western analysis using anti-hGC antibodies on 0.1 μg FLAG™ affinity-purified hGC:FLAG™. FIG. 7B. Commassie blue stained SDS-PACE gel and Western analysis of ConA-affinity-purified hGC:FLAG™. Lane 1, Coomassie blue stained SDS-PAGE gel of 10 μg of ConA purified hGC:FLAG™; Lane 2, Western analysis of ConA purified hGC:™ using anti-FLAG™ antibodies. These results indicate that the ConA-purified hGC:FLAG™ protein is glycosylated.

FIG. 8. Immuno-slot blot Western analysis using anti-FLAG™ antibodies on fractions from hGC:FLAG™ purification steps using plant tissue 12 hrs post-MGA. Lane A, FLAG™-tagged control protein: slot 1, 1 ng; slot 2, 6 ng; slot 3, 8 ng; slot 4, 18 ng; slot 5, 60 ng. Lane B, Fractions from isolation of hGC:FLAG™: slot 1, 0.5 μl/80,000 μl soluble protein from crude cell free extract; slot 2, 0.5 μl/80,000 μl soluble protein from 33% ammonium sulfate (AS) supernatant; slot 3, 2.5 μl/5,000 μl soluble protein from ConA affinity-purified hGC:FLAG™. Lane C: slot 1, 1 μl soluble protein from crude plant tissue extract; slot 2, 1 μl soluble protein from 33% AS supernatant; slot 3, 5 μl soluble protein from ConA affinity-purified hGC:FLAG™.

FIG. 9. Nucleotide sequence of hGC:FLAG™ construct (SEQ ID NO:3) which was cloned and expressed in tobacco strains X-11 and X-27. The upper case underlined letters at three positions represent changes to the sequence in GENBANK (ATCC bank cDNA sequence). The lower case letters represent additions to the hGC sequence, e.g., the FLAG™ epitope.

FIG. 10. Deduced amino acid sequence of hGC:FLAG™ fusion protein (SEQ ID NO:4). The upper case underlined letters at two positions represent changes to the original hGC amino acid sequence disclosed by E. Neufeld. Lower case letters represent additions to the hGC amino acid sequence. For example, dykddddk(SEQ ID NO:10)=the FLAG™ epitope.

FIG. 11. Sequence of 456 bases (SEQ ID NO:5) comprising the MeGA promoter.

FIG. 12. IDUA expression vector construction sctrategy. MeGA:IDUA and $35S^{ENH}$:IDUA constructs were inserted into the HindIII/SacI site of the binary vector pBIB-KAN. R and L represent T-DNA right and left borders which precisely demarcate the DNA inserted into the plant genome, NPTII is the kanamycin selectable marker, pAnos is the polyadenylation/terminator signal and Pnos a promoter from *Agrobacterium tumefaciens* nopaline synthetase gene. PCR-primers for IDUA were: ID1, (5'-CTAG tctagaATGCGTCCCCTGCGCCCCCGCG) (SEQ ID NO:6) and ID2, (5'G gaattcgagctcTCATGGATTGCCCGGGGATG) (SEQ ID NO:7); IDUA sequences are capitalized, introduced restriction sites are underlined. SP, signal peptide; IDUA, human IDUA coding region; H, HindIII; S, SacI; X, XbaI.

FIGS. 13A–C. Transgenic tobacco expressing the MeGA:IDUA construct. FIG. 13A. Germination of first generation seeds on selective medium showing segregation of kanamycin resistant and sensitive seedlings. FIG. 13B. Young plants containing the MeGA:IDUA construct (right) and untransformed parent plants grown in parallel. FIG. 13C. Fully mature IDUA-expressing plants in the greenhouse.



FIGS. 14A–B. Induction of IDUA transgene in tobacco leaf tissues. Leaf tissue from transgenic plant IDUA-9 was induced by excision into 1.5 mm strips and incubated at room temperature on moist paper towels in sealed plastic bag. Tissue was removed for analysis (stored at -80° C. for RNA, -20° C. for protein) at 0, 2, 4, 8, 11, and 27 hrs post-induction. FIG. 14A. Northern blot analysis of IDUA mRNA from transgenic tobacco plants. Fifteen μg of total RNA was run on glyoxal agarose gel, blotted onto nitrocellulose membrane, and hybridized with $^{32}$P-labeled IDUA cDNA. FIG. 14B. Western blot analysis of total soluble proteins (20 μg) from tobacco leaf extracts using antibodies to denatured IDUA synthesized in CHO cells. Control lane represents IDUA synthesized in CHO cells (98 kDa under our gel conditions). IDUA synthesized from transgenic tobacco has a molecular size of 92 kDa.

FIG. 15. Immunodetection of IDUA secreted by transgenic plants into the incubation buffer. Fifty μl of incubation buffer was boiled and slotted onto OPTITRAN membrane along with control IDUA synthesized in CHO cells. Antibodies to denatured IDUA synthesized in CHO cells were used to detect IDUA.

FIG. 16. IDUA activity in tissue extracts and incubation buffer from transgenic IDUA-9 plant tissue. Panel A: IDUA-9 plant tissue was induced and incubated in buffer, which was collected and replaced at various times after induction as described in the text. Open boxes represent IDUA activity in extracts prepared from induced tissue after incubation in buffer. Shaded boxes represent the IDUA activity in the incubation buffer. Panel B: IDUA-9 plant tissue was induced and incubated without buffer for 34 hours after which an extract was prepared from the induced tissue. The IDUA activity of the extract is shown.

FIG. 17. Comparison of IDUA activity in transgenic tobacco plants IDUA-7, IDUA-8 and IDUA-9: Panel A: Plant tissue was induced and incubated in buffer, which was collected and replaced at various times after induction as described in the text. IDUA activity present in the incubation buffer collected at various times post-indlucton was plotted. Panel B: Plant tissue was induced and incubated without buffer absence of incubation buffer for 34 hours, after which extracts were prepared from the induced tissues. The IDUA activities of the extracts are shown.

FIG. 18. Western slot blot analysis of secreted IDUA from transgenic plant IDUA-9 after three sequential addition and collection of incubation buffer; 24, 26 and 34 hrs post-MGA. The tissue (1.5 gm) was induced and incubated in a moist plastic bag for 24 hrs. Ten ml of incubation buffer was used to wash the tissue; this fraction is denoted as 24 hrs. Fresh buffer (10 ml) was added and incubated at room temperature for 2 hrs; this fraction was denoted as 26 hrs. Fresh buffer (10 ml) was added to the tissue and incubated for 8 hrs and this fraction was denoted as 34 hrs. Fifty μL of incubation buffer from each fraction was boiled and slotted onto OPTI-TRAN membrane and analyzed with anti-IDUA antibodies.

FIG. 19. The nucleotide sequence of the IDUA coding sequence (SEQ ID NO:8) used in the MeGA:IDUA and $35S^{ENH}$:IDUA expression construct.

FIG. 20. The deduced amino acid sequence (SEQ ID NO:9) of the IDUA coding sequence shown in FIG. 19.

FIG. 21. hGC cDNA plant expression construct and transformation vector. The MeGA:hGC expression construct in a pBS intermediate plasmid is excised and inserted into the SstI site of the binary plant transformation vector pBIB-KAN to form transformation vector pCT50. The PCR-amplification primers for reconstruction of the 3' end of the hGC coding region were: GC23, which has the sequence 5'GCCTATGCTGAGCACAAGTTACAG3' (SEQ ID NO:11); and GC37, whose complementary strand has the sequence 5'TTCCTTGAGCTCGTCACTGGCGACGCCA-CAGGTA3' (SEQ ID NO:12). The other abbreviations and notations shown are same as those described for FIG. 1.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the production of recombinant human or animal lysosomal enzymes in plants and in cultured plant cells and plant tissues, involving: (1) construction of recombinant expression constructs comprising lysosomal enzyme coding sequences and transformation vectors containing the expression constructs; (2) transforming or transfecting plant cells, plant tissues or plants with the transformation vectors; (3) expressing the lysosomal enzyme coding sequences in the plant cell, plant tissue or plant; and (4) detecting and purifying expression products having lysosomal enzyme activity.

The plant expression systems and the recombinant lysosomal enzymes produced therewith have a variety of uses, including but not limited to: (1) the production of enzymatically active enzymes for the treatment of lyisosomal storage diseases; (2) the production of antibodies against lysosomal enzymes, which antibodies would have medical diagnostic uses; (3) use in any commercial process that involves substrate hydrolysis; and (4) the production of modified proteins or peptide fragments to serve as precursors or substrates for further in vivo or in vitro processing to a specialized industrial form for research or therapeutic uses, such as to produce a therapeutic enzyme with increased efficacy or altered substrate specificity. These plant-expressed recombinant lysosomal protein products need not be enzymatically active or identical in structure to the corresponding native animal or human lysosomal enzymes or proteins in order to be useful for research or industrial applications.

The terms "lysosomal enzyme" and "lysosomal enzyme gene product," as used herein with respect to any such enzyme and product produced in a plant expression system, refer to a recombinant peptide expressed in a transgenic plant or plant cell from a nucleotide sequence encoding a human or animal lysosomal enzyme, a modified human or animal lysosomal enzyme, or a fragment, derivative or modification of such enzyme. Useful modified human or animal lysosomal enzymes include but are not limited to human or animal lysosomal enzymes having one or several naturally-occuring or artifically-introduced amino acid additions, deletions and/or substitutions.

The term "lysosomal enzyme coding sequence," as used herein, refers to a DNA or RNA sequence that encodes a protein or peptide, or a fragment, derivative or other modification thereof, which exhibits detectable enzymatic activity against a lysosomal enzyme substrate.

The term "enzymatically active" is used herein with respect to any recombinant lysosomal enzyme produced in a plant expression system to mean that the recombinant lysosomal enzyme is able to hydrolyze either the natural substrate, or an analogue or synthetic substrate thereof of the corresponding human or animal lysosomal enzyme, at detectable levels.

The term "enzymatically active" is also used herein with respect to recombinant hGC and modified hGC produced in a plant expression system to mean that such hGCs are able to hydrolyze the native hGC substrate, i.e., N-acyl-shingosyl-1-O-β-D-glucoside, of the hGC or that it can cleave the synthetic β-glucoside, 4-methyl-umbelliferyl-β-D-glucoside (4MuGlc), at detectable levels. Similarly, the term as applied to plant-produced IDUA and modified IDUA means that such IDUAs are able to hydrolyze the native IDUA substrate, i.e., dermatan sulfate or heparan sulfate, or is able to cleave the synthetic α-glucoside, 4-methylumbelliferyl-α-L-iduronide (4-MUI), at detectable levels.

The term "transformant" as used herein refers to a plant, plant cell or plant tissue to which a gene construct comprising a lysosomal enzyme coding sequence has been introduced by a method other than transfection with an engineered virus.

The term "transfectant" refers to a plant, plant cell or plant tissue that has been infected with an engineered virus and stably maintains said virus in the infected cell.

Once a plant transformant or transfectant is identified that expresses a recombinant lysosomal enzyme, one non-limiting embodiment of the invention involves the clonal expansion and use of that transformant or transfectant in the production and purification of enzymatically active recombinant lysosomal enzyme. In another non-limiting embodiment of the invention, each new generation of progeny plants may be newly screened for the presence of nucleotide sequence coding for a lysosomal enzyme, wherein such screening results in production by subsequent generations of plants of recoverable amounts of active recombinant lysosomal enzyme, and wherefrom the enzyme is then purified.

The invention is divided into the following sections solely for the purpose of description: (a) genes or coding sequences for lysosomal enzymes involved in lysosomal storage diseases; (b) construction of recombinant expression constructs for expressing lysosomal enzyme coding sequences in plant cell; (c) construction of plant transformation vectors comprising the expression constructs; (d) transformation/transfection of plants capable of translating and processing primary translation products in order to express an enzymatically active recombinant lysosomal enzyme; (e) identification and purification of the recombinant lysosomal enzyme so produced; (f) expansion of the number of transformed or transfected plants; and (g) methods of therapeutically using the recombinant lysosomal enzyme.

### 5.1. GENES OR CODING SEQUENCES FOR ENZYMES INVOLVED IN LYSOSOMAL STORAGE DISEASES

The recombinant lysosomal enzymes produced in accordance with this invention will have a variety of uses, probably the most significant being their use in enzyme replacement therapy for lysosomal storage diseases. These lysosomal enzymes include but are not limited to: α-N-acetylgalactosaminidase (Warner et al., Biochem. Biophys. Res. Commun., 1990, 173:13–19; acid lipase; aryl sulfatase A; aspartylglycosaminidase; ceramidase; α-L-fucosidase (de Wet et al., 1984, DNA 3:437–447), α-galactosidase, β-galactosidase, galactosylceramidase, glucocerebros:dase, α-glucosidase, β-glucuronidase, heparin N-sulfatase, β-hexosaminidase, iduronate sulfatase, α-L-iduronidase, α-mannosidase, β-mannosidase, sialidase, and sphingomyelinase. Of these enzymes, cDNAs have been cloned for α-N-acetylgalactosaminidase (Zhu & Goldstein, 1993, Gene 137:309–314); acid lipase (Amesis et al., 1994, Eur. J. Biochem 219:905–914); α-galactosidase (Eng & Desnick, 1994, Hum Mutat. 3:103–111); human glucocerebrosidase

(hGC) (Sorge et al., 1985, supra); α-L-iduronidase (Scott et al., 1991, Proc. Natl. Acad. Sci. USA 88:9695–9699); iduronate sulfatase (Daniele et al., 1993, Genomics 16:755–757); α-mannosidase (Schatzle et al., 1992, J. Biol. Chem 267:4000–4007); and sialidase (Ferrari et al., 1994, Glycobiology 4:2047–2052).

The nucleic acid sequences encoding lysosomal enzymes which can be used in accordance with the invention include but are not limited to any nucleic acid sequence that encodes a lysosomal enzyme, modified lysosomal enzyme, or functional equivalent thereof, including but not limited to: (a) any nucleotide sequence that selectively hybridizes to the complement of a human or animal lysosomal enzyme coding sequence under stringent conditions, e.g., washing in 0.1x SSC/0.1% SDS at 68° C. (Ausubel et al., eds., 1989, *Current Protocols in Molecular Biology, Vol. I*, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., New York, at page 2.10.3), and encodes a product homologous to the human or animal lysosomal enzyme; and/or (b) any nucleotide sequence that hybridizes to the complement of the human or animal lysosomal enzyme coding sequence under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42° C. (Ausubel et al., 1989, supra), yet which still encoders a homologous gene product that is enzymatically active; and (c) any nucleotide coding sequence that otherwise encodes) a protein from any organism capable of hydrolyzing a human or animal lysosomal enzyme's native substrate or substrate analogue.

The invention also includes but is not limited to: (a) DNA vectors that contain any of the foregoing nucleotide coding sequences and/or their complements; (b) DNA expression and transformation vectors that contain expression constructs comprising any of the foregoing nucleotide coding sequences operatively associated with a regulatory element that directs expression of the coding sequences in plant cells or plants; and (c) genetically engineered plant cells or plants that contain any of the foregoing coding sequences, operatively associated with a regulatory element that directs the expression of the coding and/or antisense sequences in the plant cell. As used herein, the term "regulatory element" includes but is not limited to inducible and non-induacible promoters, enhancers, operators and other elements known to those skilled in the art that drive and/or regulate gene expression. The invention also includes fragments, derivatives or other modifications of the DNA sequences described herein.

## 5.2. TRANSFORMATION VECTORS TO DIRECT THE EXPRESSION OF LYSOSOMAL ENZYME CODING SEQUENCE

### 5.2.1. LYSOSOMAL ENZYME EXPRESSION CONSTRUCTS

In order to express a lysosomal enzyme in a plant expression system, the lysosomal enzyme coding sequence is inserted into an appropriate expression construct and the expression construct is incorporated into a transformation vector for transfer into cells of the plant. The expression construct is preferably constructed so that the lysosomal enzyme coding sequence is operatively associated with one or more regulatory elements, including, e.g., promoters and/or enhancers, necessary for transcription and translation of the lysosomal enzyme coding sequence. Methods to construct the expression constructs and transformation vectors include standard in vitro genetic recombination and manipulation. See, for example, the techniques described in Weissbach and Weissbach, 1988, *Methods For Plant Molecular Biology*, Academic Press, Chapters 26–28.

Regulatory elements that may be used in the expression constructs include promoters which may be either heterologous or homologous to the plant cell. The promoter may be a plant promoter or a non-plant promoter which is capable of driving high levels transcription of a linked sequence in plant cells and plants. Non-limiting examples of plant promoters that may be used effectively in practicing the invention include cauliflower mosaic virus (CaMV) 35S, rbcS, the promoter for the chlorophyll a/b binding protein, Adhl, NOS and HMG2, or modifications or derivatives thereof. The promoter may be either constitutive or inducible. For example, and not by way of limitation, an inducible promoter can be a promoter that promotes expression or increased expression of the lysosomal enzyme nucleotide sequence after mechanical gene activation (MGA) of the plant, plant tissue or plant cell. One non-limiting example of such an MGA-inducible plant promoter is MeGA (described infra).

The expression constructs can be additionally modified according to methods known to those skilled in the art to enhance or optimize heterologous gene expression in plants and plant cells. Such modifications include but are not limited to mutating DNA regulatory elements to increase promoter strength or to alter the lysosomal enzyme coding sequence itself. Other modifications include deleting intron sequences or excess non-coding sequences from the 5' and/or 3' ends of the lysosomal enzyme coding sequence in order to minimize sequence- or distance-associated negative effects on expression of hGC, e.g., by minimizing or eliminating message destabilizing sequences.

The expression constructs may be further modifies according to methods known to those skilled in the art to add, remove, or otherwise modify peptide signal sequences to alter signal peptide cleavage or to increase or change the targeting of the expressed lysosomal enzyme through the plant endomembrane system. For example, but not by way of limitation, the expression construct can be specifically engineered to target the lysosomal enzyme for secretion, or vacuolar localization, or retention in the endoplasmic reticulum (ER).

In one embodiment, the expression construct can be engineered to incorporate a nucleotide sequence that encodes a signal targeting the lysosomal enzyme to the plant vacuole. For example, and not by way of limitation, the N-terminal 143 amino acid domain derived from the plant vacuolar protein, proaleurain (Holwerda et al., 1992, supra; Holwerda et al., 1990, supra), may be engineered into the expression construct to produce a signal peptide-lysosomal enzyme fusion product upon transcription and translation. The proaleurain signal peptide will direct the lysosomal enzyme to the plant cell vacuole, but is itself cleaved off during transit through the plant endomembrane system to generate the mature protein.

In another non-limiting embodiment, a signal peptide may be engineered into the expression construct to direct the lysosomal enzyme to be secreted from the plant cell. For example, and not by way of limitation, the signal peptide of tobacco PR-1, which is a secreted pathogenesis-related protein (Cornelissen et al., 1986, EMBO J. 5:37–40), can be engineered into the expression construct to direct the secretion of the lysosomal enzyme from the plant cell.

In an additional non-limiting embodiment, the signal peptide may be engineered into the expression construct to direct the lysosomal enzyme to be retained within the ER. Such ER-retained lysosomal enzymes may exhibit altered, and perhaps preferable, glycosylation patterns as a result of failure of the peptide to progress through the Golgi

apparatus, thus resulting in a lack of subsequent glycosyl processing. For example, and not by way of limitation, a nucleotide sequence can be engineered into the expression construct to result in fusion of the amino acid sequence KDEL (SEQ ID NO:15), i.e., Lys-Asp-Glu-Leu, to the carboxyl-terminus of the lysosomal enzyme. The KDEL sequence results in retention of the lysosomal enzyme in the ER (Pfeffer and Rothman, 1987, Ann. Rev. Biochem. 56:829–852).

Expression construct may be further modified according to methods known to those skilled in the art to add coding sequences that facilitate purification of the lysosomal enzyme. In one non-limiting embodiment, a nucleotide sequence coding for the target epitope of a monoclonal antibody may be engineered into the expression construct in operative association with the regulatory elements and situated so that the expressed epitope is fused to the lysosomal enzyme. For example, and not by way of limitation, a nucleotide sequence coding for the FLAG™ epitope tag (International Biotechnologies, Inc., IBI), which is a hydrophilic marker peptide, can be inserted by standard techniques into the expression construct at a point corresponding to the carboxyl-terminus of the lysosomal enzyme. The expressed FLAG™ epitope-lysosomal enzyme fusion product may then be detected and affinity-purified using anti-FLAG™ antibodies.

In another non-limiting embodiment, a nucleotide sequence can be engineered into the expression construct to provide for a cleavable linker sequence between the lysosomal enzyme peptide sequence and any targeting signal, reporter peptide, selectable marker, or detectable marker, as described supra, that has not otherwise been cleaved from the lysosomal enzyme peptide sequence during peptide processing and trafficking through the plant endomembrane system. Such a linker sequence can be selected so that it can be cleaved either chemically or enzymatically during purification of the lysosomal enzyme (Light et al., 1980, Anal. Biochem. 106:199–206).

5.2.2. PLANT TRANSFORMATION VECTORS

The transformation vectors of the invention may be developed from any plant transformation vector known in the art include, but are not limited to, the well-known family of Ti plasmids from Agrobacterium and derivatives thereof, including both integrative and binary vectors, and including but not limited to pBIB-KAN, pGA471, pEND4K, pGV3850, and pMON505. Also included are DNA and RNA plant viruses, including but not limited to CaMV, geminiviruses, tobacco mosaic virus, and derivatives engineered therefrom, any of which can effectively serve as vectors to transfer a lysosomal enzyme coding sequence, or functional equivalent thereof, with associated regulatory elements, into plant cells and/or autonomously maintain the transferred sequence. In addition, transposable elements may be utilized in conjunction with any vector to transfer the coding sequence and regulatory sequence into a plant cell.

To aid in the selection of transformants and transfectants, the transformation vectors may preferably be modified to comprise a coding sequence for a reporter gene product or selectable marker. Such a coding sequence for a reporter or selectable marker should preferably be in operative association with the regulatory element coding sequence described supra.

Reporter genes which may be useful in the invention include but are not limited to the β-glucuronidase (GUS) gene (Jefferson et al., 1986, Proc. Natl. Acad. Sci. USA, 83:8447), and the luciferase gene (Ow et al., 1986, Science 234:856). Coding sequences that encode selectable markers which may be useful in the invention include but are not limited to those sequences that encode gene products conferring resistance to antibiotics, anti-metabolites or herbicides, including but not limited to kanamycin, hygromycin, streptomycin, phosphinothricin, gentamicin, methotrexate, glyphosate and sulfonylurea herbicides, and include but are not limited to coding sequences that encode enzymes such as neomycin phosphotransferase II (NPTII), chloramphenicol acetyltransferase (CAT), and hygromycin phosphotransferase I (HPT, HYG).

5.3. TRANSFORMATION/TRANSFECTION OF PLANTS

A variety of plant expression systems may be utilized to express the lysosomal enzyme coding sequence or its functional equivalent. Particular plant species may be selected from any dicotyledonous, monocotyledonous species, gymnospermous, lower vascular or non-vascular plant, including any cereal crop or other agriculturally important crop. Such plants include, but are not limited to, alfalfa, Arabidopsis, asparagus, barley, cabbage, carrot, celery, corn, cotton, cucumber, flax, lettuce, oil seed rape, pear, peas, petunia, poplar, potato, rice, soybean, sugar beet, sunflower, tobacco, tomato, wheat and white clover.

Methods by which plants may be transformed or transfected are well-known to those skilled in the art. See, for example, *Plant Biotechnology*, 1989, Kung & Arntzen, eds., Butterworth Publishers, ch. 1, 2. Examples of transformation methods which may be effectively used in the invention include but are not limited to Agrobacterium-mediated transformation of leaf discs or other plant tissues, microinjection of DNA directly into plant cells, electroporation of DNA into plant cell protoplasts, liposome or spheroplast fusion, microprojectile bombardment, and the transfection of plant cells or tissues with appropriately engineered plant viruses.

Plant tissue culture procedures necessary to practice the invention are well-known to those skilled in the art. See, for example, Dixon, 1985, *Plant Cell Culture: A Practical Approach*, IRL Press. Those tissue culture procedures that may be used effectively to practice the invention include the production and culture of plant protoplasts and cell suspensions, sterile culture propagation of leaf discs or other plant tissues on media containing engineered strains of transforming agents such as, for example, Agrobacterium or plant virus strains and the regeneration of whole transformed plants from protoplasts, cell suspensions and callus tissues.

The invention may be practiced by transforming or transfecting a plant or plant cell with a transformation vector containing an expression construct comprising a coding sequence for the lysosomal enzyme and selecting for transformants or transfectants that express the lysosomal enzyme. Transformed or transfected plant cells and tissues may be selected by techniques well-known to those of skill in the art, including but not limited to detecting repo-ter gene products or selecting based on the presence of one of the selectable markers described supra. The transformed or transfected plant cells or tissues are then grown and whole plants regenerated therefrom. Integration and maintenance of the lysosomal enzyme coding sequence in the plant genome can be confirmed by standard techniques, e.g., by Southern hybridization analysis, PCR analysis, including reverse transcriptase-PCR (RT-PCR), or immunological assays for the expected protein products. once such a plant transformant or transfectant is identified, a non-limiting embodiment of the invention involves the clonal expansion and use of that transformant or transfectant in the production of lysosomal enzyme.

As one non-limiting example of a transformation procedure, Agrobacterium-mediated transformation of plant

leaf disks can follow procedures that are well known to those skilled in the art. Briefly, leaf disks can be excised from axenically grown plant seedlings, incubated in a bacterial suspension, for example, $10^9$ cfu/ml, of *A. tumefaciens* containing an engineered plasmid comprising a selectable marker such as, for example, kanamycin resistance, and transferred to selective "shooting" medium containing, for example, kanamycin, that will block growth of bacteria and untransformed plant cells and induce shoot initiation and leaf formation from transformed cells. Shoots are regenerated and then transferred to selective media tc trigger root initiation. Stringent antibiotic selection at the rooting step is useful to permit only stably transformed shoots to generate roots. Small transgenic plantlets may then be transferred to sterile peat, vermiculite, or soil and gradually hardened off for growth in the greenhouse or in the field.

5.4. IDENTIFICATION AND PURIFICATION OF THE LYSOSOMAL ENZYME GENE PRODUCT

Transcription of the lysosomal enzyme coding sequence and production of the lysosomal enzyme in transformed or transfected plants, plant tissues, or plant cells can be confirmed and characterized by a variety of methods known to those of skill in the art. Transcription of the lysosomal enzyme coding sequence can be analyzed by standard techniques, including but not limited to detecting the presence of lysosomal enzyme messenger ribonucleic ac(id (mRNA) transcripts in transformed or transfected plants or plant cells using Northern hybridization analysis or RT-PCR amplification.

Detection of the lysosomal enzyme itself can be carried out using any of a variety of standard techniques, including, but not limited to, detecting lysosomal enzyme activity in plant extracts, e.g., by detecting hydrolysis either of the enzyme's natural substrate or a substrate analogue. Additionally, the lysosomal enzyme can be detected immunologically using monoclonal or polyclonal antibodies, or immuno-reactive fragments or derivatives thereof, raised against the enzyme, e.g., by Western blot analysis, and limited amino acid sequence determination of the protein.

Indirect identification of enzyme production in a plant can be performed using any detectable marker or reporter linked to the lysosomal enzyme. For example, but not by way of limitation, the FLAG™ epitope, which can be linked to the lysosomal enzyme, as described supra, is detectable in plant tissues and extracts using anti-FLAG M2 monoclonal antibodies (IBI) in conjunction with the Western Exposure™ chemi-luminescent detection system (Clontech).

Lysosomal enzyme production in a transformed or transfected plant can be confirmed and further characterized by histochemical localization, the methods of which are well-known to those skilled in the art.. See, for example, *Techniques in Immunocytochemistry, Vol I*, 1982, Bullock and Petrusz, eds., Academic Press, Inc. For example, but not by way of limitation, either fresh, frozen, or fixed and embedded tissue can be sectioned, and the sections probed with either polyclonal or monoclonal primary antibodies raised against the lysosomal enzyme or, for example, anti-FLAG™ monoclonal antibodies. The primary antibodies can then be detected by standard techniques, e.g., using the biotinylated protein A-alkaline phosphatase-conjugated streptavidin technique, or a secondary antibody bearing a detectable label that binds to the primary antibody.

The expression products can be further purified and characterized as described in the subsections below.

5.4.1. PRODUCTION AND PURIFICATION OF THE LYSOSOMAL ENZYME GENE PRODUCT

One non-limiting method to produce and purify the lysosomal enzyme is described here, wherein the lysosomal enzyme coding sequence is operably associated with an inducible promoter in the expression construct. Leaf or other tissue or cells from a transgenic plant or cell culture transformed or transfected with this expression construct can be processed to induce expression of the lysosomal enzyme coding sequence. This induction process may include inducing the activation of lysosomal genes by one or more methods, applied separately or in combination, including but not limited to physical wounding or other mechanical gene activation (MGA), and application of chemical or pathogenic elicitors or plant hormones. Lysosomal gene activation levels may also be enhanced in plant cells or tissues by factors such as the availability of nutrients, gases such as $O_2$ and $CO_2$, and light or heat. After induction of expression, the tissue can be stored, e.g., at −20° C. If the lysosomal protein is targeted for localization within the plant cell, the plant cell wall must be penetrated to extract the protein. Accordingly, the plant tissue can be ground to a fine powder, e.g., by using a tissue grinder and dry ice, or homogenized with a ground glass tissue homogenizer. To resuspend the lysosomal enzyme, plant membranes must be solubilized using an extraction buffer containing a detergent, e.g., a bile detergent such as 1% (w/v) sodium taurocholate, in a buffered solution, e.g., 25 mM sodium citrate, pH 7.0. The homogenate can then be clarified by, for example, centrifugation at 10,000×g for 30 min to produce a cell-free homogenate.

The lysosomal enzyme must be further purified ilf it is to be useful as a therapeutic or research reagent. The lysosomal enzyme can be purified from plant extracts according to methods well-known to those of skill in the art (Furbish et al., 1977, Proc. Natl. Acad. Sci. USA 74:3560–3563). Once the presence of the enzyme is confirmed it can be isolated from plant extracts by standard biochemical techniques including, but not limited to, differential ammonium sulfate (AS) precipitation, gel filtration chromatography or affinity chromatography, e.g., utilizing hydrophobic, immunological or lectin binding. At each step of the purification process the yield, purity and activity of the enzyme can be determined by one or more biochemical assays, including but not limited to: (1) detecting hydrolysis of the enzyme's substrate or a substrate analogue; (2) immunological analysis by use of an enzyme-linked immunosorbent assay (ELISA); (3) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis; and (4) Western analysis. The enzyme may be alternatively or additionally purified by affinity chromatography wherein the enzyme binds to its inhibitor which is linked, for example, to an inert substrate.

Once solubilized, all enzyme-containing fractions can be maintained, for example, by storage at 4° C., and stabilized if necessary, e.g., with 4 mM β-mercaptoethanol, 5 mM EDTA, and/or possibly with high levels of glycerol or ethylene glycol.

5.4.2. PROTEOLYTIC PROCESSING OF THE SIGNAL PEPTIDE

In order to address whether the plant expression system efficiently recognizes and correctly cleaves the human signal peptide from the lysosomal enzyme, the plant-produced enzyme can be purified and analyzed by N-terminal sequencing. Accordingly, the enzyme can, for example, be treated with Endo-F/N-glucanase (Boehringer Mannheim) to remove N-linked glycans, and the resulting peptide can be repurified by methods described supra. The purity of the enzyme can be determined based, for example, on silver-stained SDS-PAGE. The band containing the enzyme can be excised from the gel, the peptide eluted therefrom, and then analyzed by commercial N-terminal amino acid sequencing to determine whether the correct cleavage of the signal

peptide has occurred. Incomplete cleavage can be detected, for example, as a double band on SDS-PAGE, or as mixed N-terminal sequences.

5.4.3. N-LINKED GLYCOSYLATION IN PLANTS VERSUS ANIMALS

The oligosaccharides of native human and animal lysosomal enzymes are typical antennary structures containing N-acetylglucosamine, mannose, and sialic acid. The glycoconjugate associated with the lysosomal enzyme of the invention may be determined, for example, by lectin binding studies (Reddy et al., 1985, Biochem. Med. 33:200–210, Cummings, 1994, Meth. Enzymol. 230:66–86).

Plant glycans do not contain sialic acid, which is a prevalent terminal sugar in mammalian glycans. In addition, the complex glycans of plants are generally smaller and contain a $\beta$1-2 xylose residue attached to the $\beta$-linked mannose residues of the core (Gomez and Chrispeels, 1994, Proc. Natl. Acad. Sci. USA 91:1829–1833).

Determination of the glycan composition and structure of the lysosomal enzyme of the invention is of particular interest because: (a) the glycan composition will indicate the status of the protein's movement through the Golgi; and (b) the presence of a complex glycan may indicate whether an antigenic response will be triggered in humans.

Several molecular, genetic and chemical approaches can be used to raise the proportion of the high-mannose form of glycans on lysosomal enzymes, making them more similar in structure to the native human protein (Grabowski et al., 1995, Ann. Int. Med. 122:33–39; Berg-Fussman et al., 1993, J. Biol. Chem. 268:14861–14866). For example, but not by way of limitation, the mannose analog, 1-deoxymannojirimycin (dMM), inhibits mannosidase I, the first Golgi-specific enzyme involved in glycan processing. Plant tissues treated with dMM produce glycoproteins which lack fucose and xylose and maintain a glycan profile consistent with inhibition at the mannosidase I step (Vitale et al., 1989, Pl. Phys. 89:1079–1084). Treatment of lysosomal enzyme-expressing plant tissues with dMM may be useful to produce lysosomal enzymes with a relatively homogeneous high-mannose glycan profile. Such lysosomal enzymes should be highly effective for use in treatment of lysosomal storage diseases in human and animals.

5.5. CLONAL PROPAGATION AND BREEDING OF TRANSGENIC PLANTS

Once a transformed or transfected plant is selected that produces a useful amount of the recombinant lysosomal enzyme of the invention, one embodiment of the invention contemplates the production of clones of this plant either by well-known asexual reproductive methods or by standard plant tissue culture methods. For example, tissues from a plant of interest can be induced to form genetically identical plants from asexual cuttings. Alternatively, callus tissue and/or cell suspensions can be produced from such a plant and subcultured. An increased number of plants can subsequently be regenerated therefrom by transfer to the appropriate regenerative culture medium.

Alternatively, the recombinant lysosomal enzyme-producing plant may be crossed as a parental line, either male or female, with another plant of the same species or variety, which other plant may or may not also be transgenic for the lysosomal coding sequence, to produce an F1 generation. Members of the F1 and subsequent generations can be tested, as described supra, for the stable inheritance and maintenance of the lysosomal enzyme coding sequence, as well as for lysosomal enzyme production. A breeding program is thus contemplated whereby the lysosomal enzyme coding sequence may be transferred into other plant strains or varieties having advantageous agronomic characteristics, for example, by a program of controlled backcrossing. The invention thus encompasses parental lines comprising the lysosomal enzyme coding sequence, as well as all plants in subsequent generations descending from a cross in which at least one of the parents comprised the lysosomal enzyme coding sequence. The invention further encompasses all seeds comprising the lysosomal enzyme coding sequence and from which such plants can be grown, and tissue cultures, including callus tissues, cell suspensions and protoplasts, comprising the lysosomal enzyme coding sequence, whether or not they can be regenerated back to plants.

5.6. METHODS FOR THERAPEUTIC USE OF LYSOSOMAL ENZYMES

The recombinant lysosomal enzymes of the invention are useful for therapeutic treatment of lysosomal storage diseases by providing a therapeutic amount of a particular lysosomal enzyme, or a derivative or modification thereof, to a patient suffering from a lysosomal storage disease or condition resulting from a deficiency of the corresponding human or animal active form of that enzyme.

By "therapeutic amount" is meant an amount of enzymatically active lysosomal enzyme which will cause significant alleviation of clinical symptoms of a particular lysosomal storage disease.

A therapeutic amount causes "significant alleviation of clinical symptoms" of the particular lysosomal storage disease if it serves to reduce one or more of the pathological effects or symptoms of the disease or to reduce the rate of progression of one or more of such pathological effects or symptoms.

An effective dosage and treatment protocol may be determined by conventional means, starting with a low dose in laboratory animals and then increasing the dosage while monitoring the effects, and systematically varying the dosage regimen as well. The amount of recombinant lysosomal enzyme to be administered to a patient suffering from a lysoc;omal disease or condition will vary. Numerous factors may be taken into consideration by a clinician when determining an optimal dose for a given subject. These factors include the size of the patient, the age of the patient, the general condition of the patient, the particular disease being treated, the severity of the disease, the presence of other drugs in the patient, and the like. Trial dosages would be chosen after consideration of the results of animal studies, and any available clinical literature with respect to past results of replacement therapy for the particular lysosomal storage disease.

For example, therapeutic amounts of recombinant hGC and IDUA and modified hGC and IDUA produced according to the invention may in each instance encompass dosages of between about 10 and about 500 mg per 70 kg patient per month, depending upon the severity of the patient's symptoms of the Gaucher's or Hurler's disease.

The amount of recombinant lysosomal enzyme of the invention administered to the patient may be decreased or increased according to the enzymatic activity of the particular lysosomal enzyme. For example, administration of a recombinant lysosomal enzyme of the invention which has been modified to have increased enzymatic activity relative to the native human or animal enzyme will require administration of a lesser amount to the patient than a native human or animal lysosomal enzyme having lower enzymatic activity.

In addition, the amount of recombinant lysosomal enzyme administered to the patient may be modified over time

depending on a change in the condition of the patient as treatment progresses, the determination of which is within the skill of the attending clinician.

The invention also provides pharmaceutical formulations for use of the recombinant lysosomal enzyme in treating lysosomal storage diseases. The formulations comprise a recombinant lysosomal enzyme of the invention and a pharmaceutically acceptable carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, and the like. The pharmaceutical formulations may also comprise additional components that serve to extend the shelf-life of pharmaceutical formulations, including preservatives, protein stabilizers, and the like. The formulations are preferably sterile and free of particulate matter (for injectable forms). These compositions may be sterilized by conventional, well-known sterilization techniques.

The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents and the like, e.g., sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc.

The formulations may be adapted for various forms of administration, including intramuscularly, subcutaneously, intravenously and the like. The subject formulations may also be formulated so as to provide for the sustained release of a lysosomal enzyme. Actual methods for preparing parenterally administrable compositions and adjustments necessary for administration to subjects will be known or apparent to those skilled in the art and are described in more detail in , for example, Remington's *Pharmaceutical Science, 17th Ed.*, Mack Publishing Company, Easton, Pa. (1985), which is incorporated herein by reference.

The invention is illustrated in the working examples described infra, for the expression of hGC in tobacco.

## 6. EXAMPLE 1

## PRODUCTION AND ISOLATION OF RECOMBINANT MODIFIED hGC FROM TRANSGENIC TOBACCO PLANTS

The subsections below describe the production of an enzymatically active modified human glucocerebrosidase (hGC) in tobacco.

### 6.1. CONSTRUCTION OF A MODIFIED hGC EXPRESSION CONSTRUCT AND INSERTION INTO A PLANT TRANSFORMATION VECTOR

### 6.1.1. PROMOTER:hGC EXPRESSION CONSTRUCT

*E. coli* containing the hGc cDNA sequence cloned from fibroblast cells, as described (Sorge et al., 1985, supra), was obtained from the ATCC (Accession No. 65696). Oligonucleotide primers GC1 (corresponding to the amino terminus of the hGC coding region as shown in FIG. 1), and GC4 (corresponding to the carboxy terminus of the hGC coding region), were used to amplify the hGC cDNA sequence using the polymerase chain reaction (PCR). Primer GC1 was designed to include the hGC ATG initiation codon and to generate a 5' XbaI site. Primer GC4, complementary to hGC mRNA, does not include the stop codon for the gene and was designed to generate an EcoRI restriction site. The design of oligonucleotide GC4 also corrected an altered base in the ATCC sequence (GenBank/EMBL #M11080), thus producing an Arg-Arg-Gln sequence upstream to the site where a FLAG™ epitope will be inserted.

The 1.9 kb fragment generated by PCR was purified by agarose gel elution, digested with XbaI and EcoRI, and ligated into the similarly digested plasmid, Bluescript SK$^-$ (Stratagene). This cloning vector was chosen because of its small size (2.9 kb) and its extensive multiple cloning region.

The MeGA promoter, comprising a 456 bp fragment (FIG. 11) (SEQ ID NO:5) as modified from the tomato HMG2 promoter (Weissenborn et al., 1995, Phys. Plantarum 93:393–400), was used to drive the expression of the hGC gene. The MeGA promoter is inducible and has a low basal expression in unstressed plant tissues, but is highly induced in both immature and mature tissues by the process of mechanical gene activation (MGA), or by a variety of chemicals that induce plant defense responses. MGA includes but is not limited to the mechanical shredding of leaf tissue, for example, into 2 mm strips, followed by storage at room temperature on Whatman 3 MM chromatography paper moistened with sterile water in a sealed plastic bag. The expression of a MeGA:GUS construct has been monitored in transgenic tobacco plants from seedling stage to flowering and it showed no loss of inducible activity as plants reached maturity.

The 456 bp MeGA promoter was PCR-amplified using primers which incorporated a NotI restriction site at the 5' end of the fragment and a XbaI site at the 3' end of the promoter. This fragment also contained the 5'-untranslated leader of its native tomato sequence and thus provided all necessary 5' elements for expression of the fused hGC sequences. Following amplification, the fragment was PAGE-purified, digested with NotI and XbaI, and ligated into the plasmid containing the hGC coding region, which had also been NotI/XbaI digested, to produce a MeGA:hGC fusion.

### 6.1.2. GENERATION OF A MeGA:hGC:FLAG™ CONSTRUCT

In order to facilitate detection and purification of the hGC gene product, a FLAG™ epitope coding sequence was fused in frame to the C-terminus of the hGC coding sequence. The FLAG™ epitope (IBI) is the octapeptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (or DYKDDDDK) (SEQ ID NO:10) designed to be a hydrophilic marker peptide situated on a protein surface to facilitate antibody interactions (Shelness, 1992, Epitope 1:11–17; Hopp et al., 1988, Bio/Tech. 6:1204–1210).

A double-stranded oligonucleotide (FIG. 1) was synthesized which incorporated: (a) a 5' EcoRI restriction site which creates an in-frame fusion with the engineered hGC C-terminus EcoRI site; (b) the FLAG™ octapeptide coding region; (c) a stop codon following the epitope; and (d) a 3' SstI/EcoRI site. The DNA encoding FLAG™ was PAGE-purified, digested with EcoRI, and the fragment encoding FLAG™ inserted into the EcoRI site of the MeGA:hGC plasmid, and tested for insert orientation.

The translational fusion was tested by in vitro transcription using T3 RNA polymerase driven by the T3 promoter in the pBluescript SK- vector following excision of the MeGA promoter, and in vitro translation in the presence of $^{35}$S-methionine using rabbit reticulocyte lysates (BRL). The major translation product was about 56–59 kDa, consistent with the expected size of the hGC:FLAG™ fusion product (59 kDa). In addition, the hGC:FLAG™ fusion construct was completely sequenced using the dideoxy-sequenase system (USB). The nucleotide sequence of the hGC:FLAG™ fusion (SEQ ID NO:3) is shown in FIG. 9; the deduced amino acid sequence (SEQ ID NO:4) is shown in FIG. 10. The construction altered amino acid residue 545 to an arginine (R) and added ten amino acid residues, including the FLAG™ octapeptide, to the carboxyterminal of hGC. See FIG. 10.

6.1.3. INSERTION OF THE MeGA:hGC:FLAG™ CONSTRUCT INTO A PLANT TRANSFORMATION VECTOR

The MeGA:hGC:FLAG™ expression construct was excised from the pBluescript vector by digestion with SstI and ligated into the corresponding restriction site in the multiple cloning region of the plant binary vector pBIB-KAN (Becker, 1990, Nucl. Acids Res. 18:203) to form plasmid CTProl:hGC:FLAG™. As shown in FIG. 1, insertion of the MeGA:hGC:FLAG™ expression construct correctly positioned a plant transcriptional terminator for the construct. In addition, the binary vector carries an NPTII gene within the transfer DNA (T-DNA) which allows for selection of transformed plant cells based on kanamycin resistance. The engineered plasmid was transformed into *E. coli* strain DH5α and tested for correct insertion prior to mobilization into *Agrobacterium tumefaciens* strain LBA4404 (Hoekma et al., 1983, Nature 303:179–180).

6.2. INTRODUCTION OF THE MeGA:hGC:FLAG™ EXPRESSION CONSTRUCT INTO TOBACCO AND ASSESSMENT OF hGC:FLAG™ EXPRESSION

6.2.1. GENERATION OF TRANSGENIC TOBACCO PLANTS CONTAINING THE MeGA:hGC:FLAG™ CONSTRUCT

Agrobacterium-mediated transformation (Horsch et al., 1984, Science 223:496–498) was used to stably integrate the modified T-DNA sequence containing the MeGA:hGC:FLAG™ construct into the genome of tobacco. Leaf discs excised from aseptically grown seedlings of tobacco (*Nicotiana tabacum*) cvs. Xanthi (a non-commercial variety) and VA116 (a commercial, flue-cured variety) were briefly incubated in a bacterial suspension ($10^9$ cfu/ml) of *A. tumefaciens* containing the engineered plasmid (FIG. 2A), and co-cultivated on plates containing a nurse-culture of cultured tobacco cells for 48 hr. The leaf discs were then transferred to MS media (Murashige & Skoog, 1962, Physiol. Plant. 15:473–497) containing 100 mg/L kanamycin and 9.12 μM zeatin, which is a selective "shooting" medium that blocks the growth of bacteria and untransformed plant cells, and encourages shoot formation (Horsch et al., supra).

Shoots were observed three weeks post-inoculation (FIG. 2B) and were excised and placed on selective rooting media (100 mg/L kanamycin, 10 μM indole-3-acetic acid in MS media). After 1 week, the rooted plantlets (FIG. 2C) were transferred to sterile potting soil and placed in the greenhouse (FIG. 2D). Additional shoots were excised and rooted over the next 4 weeks with a total of 45 individual transformants being brought to soil (FIG. 2E). The presence of the gene construct did not appear to have any effect on the growth or development of these transformants.

6.2.2. SOUTHERN ANALYSIS OF MeGA:hGC:FLAG™ INSERTIONS IN TRANSGENIC PLANTS

The stable insertion of the MeGA:hGC:FLAG™ construct was confirmed by genomic Southern hybridization analysis. Total DNA was isolated from leaf tissue of eight young regenerants and digested with HindIII, which cuts only once within the introduced DNA (see FIG. 1). The second HindIII site flanks the introduced DNA and is located within the plant's genomic DNA. Thus, when probed with hGC cDNA sequences (1.7 kb HindIII fragment from pBluescript intermediate vector) 3' of the HindIII site, each fragment should be a distinctive size and represent an independent insertional event within the plant genome.

Five of the eight putative transformants tested showed multiple hGC inserts (FIG. 3). Four of these plants (X-1, X-8, X-9 and X-11) were derived from the Xanthi cultivar. One plant (V-1) was derived from cultivar VA116. Transformant X-8 had less DNA loaded and showed two bands upon longer autoradiographic exposure. In addition, high levels of hGC were detected in other transformants for which Southern hybridizations were not carried out, including a plant designated X-27.

6.2.3. NORTHERN ANALYSIS OF TRANSCRIPTIONAL ACTIVATION OF THE MeGA:hGC:FLAG™ TRANSGENE

As described supra, the MeGA promoter is essentially inactive in unstressed leaves, but is activated by MGA (see FIG. 4) or by treatment with chemicals that induce plant defense responses. In order to demonstrate that transgenic plants express hGC:FLAG™ mRNA in the expected inducible expression pattern, transformed plant tissue was induced by MGA, i.e., by shredding the leaf tissue into 2 mm strips, followed by incubation of Whatman #1 paper moistened with sterile water within a ZipLoc™ plastic bag and incubated at room temperature for 24 hrs. Total RNA was isolated by standard guanidino-thiocyanate methods from leaf tissue of untransformed and transformed plants immediately upon excision (time 0), or at 24 hr after MGA.

As shown in FIG. 4, hGC:FLAG™ mRNA levels were undetectable in leaves of X-11 at the time 0, but showed a marked increase in hGC transcript levels 24 hr after MGA. A more detailed time course of a second plant, V-1, showed detectable mRNA by 4 hr, maximal RNA levels at 24 hr, and mRNA levels declining at 48 hr. In addition, transcript levels increased in response to chemical defense elicitors compared to MGA. This pattern of expression is exactly that expected of a transgene construct linked to the MeGA promoter (Park et al., 1992, Pl. Mol. Biol. 20:327–331; Yang et al., 1991, Pl. Cell 3:397–405).

6.2.4. IMMUNODETECTION OF THE hGC:FLAG™ PROTEIN IN TRANSGENIC PLANT EXTRACTS

As described supra, the hGC:FLAG™ fusion construct was designed to utilize the FLAG™ epitope to facilitate detection and purification of the hGC:FLAG™ fusion protein. Seven weeks after plants were potted in soil, leaf discs from 35 plants of the 45 transformants described above were harvested (and thereby wounded) to induce transgene expression.

Extracts from the leaf discs of control plants and transgenic plants were spotted on nitrocellulose membranes for immuno-dot blot analysis. Monoclonal antibodies (anti-FLAG M2, IBI) against the FLAG™ epitope, in conjunction with the Western Exposure™ chemiluminescent detection system (Clontech, Inc.), were used to test for immunoreactive material. Of the 35 plants tested, 25 showed significant transgene expression.

Western analysis of extracts from wounded leaves of untransformed plants and transformed plants were tested for immuno-reactivity to polyclonal antibodies raised against hGC (FIG. 5B). These antibodies have not shown binding to any mammalian proteins other than the acid β-glucosidase, i.e., glucocerebrosidase of chimpanzees. Extracts from transgenic plants showed strong immuno-reactivity by a single protein band with an apparent molecular weight of about 66–69 kDa (FIG. 5B). The size of the immunoreactive protein wias reduced to about 58 kDa after N-glucanase treatment, indicating that the enzyme was glycosylated. Analogous Western immunoblots probed with anti-FLAG™ antibodies showed additional similar molecular weight bands (FIG. 5A), suggesting that both the polyclonal antibody to hGC and the anti-FLAG™ antibody recognize the same fusion protein product.

6.2.5. ENZYMATIC ACTIVITY IN TOBACCO EXTRACTS

Plant tissues were tested for hGC activity using a sensitive and convenient assay that is widely utilized in Gaucher disease research (Grabowski et al., 1990, in: *Critical Reviews in Biochemistry and Molecular Biology*, 25:385–414, CRC Press, Inc.). This assay uses the fluorometric substrate, 4-methylumbelliferyl-β-D-glucopyranoside (4MuGlc) (the "4MuGlc assay"). An increase in absorbance at 460 nm results from cleavage of 4MuGlc, and indicates the presence of enzymatic activity. 4MuGlc also serves as a substrate for endogenous plant β-glucosidases which have been detected in leaves of both control and transgenic plants. However, several distinctive properties of hGC were used to distinguish between endogenous glucosidase activity and hGC activity (TABLE 1). The differences in solubility together with the use of anti-FLAG™ affinity system for purification of the hGC-:FLAG™ were employed to solve the problem of separating hGC:FLAG™ from the endogenous plant β-glucosidases (Table 2, FIG. 8).

TABLE 1

Comparisons of endogenous tobacco β-glucosidase and hGC:FLAG™

| CHARACTERISTICS | ENDOGENOUS | hGC:FLAG™ |
|---|---|---|
| Solubility | Present in soluble extract in 0.1% Triton X-100 buffer | Membrane-associated, requiring high Triton concentration, sonication, or freeze/thaw to solubilize |
| Response to MGA | High levels in unstressed leaves, declines approx. 80% post-MGA | Absent in unstressed leaves, induced 24–48 hrs post-MGA |
| Inhibition | Weakly inhibited by conduritol B epoxide (CBE) (Sigma) | Strongly inhibited by CBE |
| Substrate | Active with MuGlc | Active with MuGlc |
| Antibody response | No immuno-reactivity to anti-FLAG™ or anti-hGCase antibodies | Immuno-reactive to both anti-FLAG™ and anti-hGCase antibodies |

6.2.6. ACCUMULATION OF hGC:FLAG™ PROTEIN IN TOBACCO TISSUES

In order to determine the best length of incubation time post-MGA for optimum yield of hGC:FLAG™ protein and hGC enzyme activity, extracts were analyzed from transgenic leaves at 0, 2, 4, 8, 16, and 24 hrs post-MGA. Plant tissue (0.5 gm) was ground using dry ice and a coffee bean grinder. To solubilize hGC:FLAG™, the ground tissue was resuspended in 1.0 ml of extraction buffer containing 25 mM sodium citrate pH 7.0, 1% (w/v) sodium taurocholate, 4 mM β-mercaptoethanol, and 5 mM EDTA. The homogenate was frozen in a dry ice/ethanol bath for 30 min and thawed at 4° C. for 2 hrs. This freeze-thaw procedure was repeated. Cell debris was pelleted at 14,000xg for 15 min. at 4° C. The cell free supernatant was collected and brought up to 40% (v/v) glycerol in order inhibit the denaturation of hGC:FLAG™ protein.

Western analysis was carried out on 10 μg of soluble protein from leaf extracts to test for immuno-reactivelty to polyclonal antibodies raised against hGC (FIG. 5B) and monoclonal antibodies against the FLAG™ epitope (FIG. 5A). The highest level of induction of hGC:FLAG™ protein occurred between 8 and 12 hrs post-MGA.

To determine the optimum time post-MGA for obtaining the highest level of hGc enzymatic activity, 0.1 μg of leaf extracts were assayed using the 4MuGlc assay. The highest hGC activity was found in extracts from 12 hrs post-wounded tissue (FIG. 6).

6.3. PURIFICATION OF hGC:FLAG™ FROM TOBACCO EXTRACTS

Forty gms of post-wounded (12 hrs) tissue was ground to a fine powder using dry ice and a coffee bean grinder. One hundred mls of extract buffer were added and the sample was made into a slurry using a polytron (Brinkman Scientific). The extract was frozen in a dry ice/ethanol bath for 1 hr and thawed for 16 hrs at 4° C. Cell debris was pelleted at 14,000xg for 30 min. The supernatant was filtered through 8 layers of cheese cloth and the filtrate was saved. An 1 ml aliquot was stored in 40% (v/v) glycerol for later protein and hGC enzymatic activity determination, while ammonium sulfate (AS) was gradually added with stirring to the remaining filtrate to 33% (w/v) final concentration and incubated at 4° C. for 1 hr. The homogenate was cleared by centrifugation at 14,000xg for 30 min. The supernatant was dialyzed overnight at 4° C. against the following buffer: 0.1M sodium citrate, pH 6.0, 4 mM β-mercaptoethanol and 5 mM EDTA. The supernatant was clarified by centrifugation at 14,000xg for 30 min. The cleared supernatant was concentrated (Amicon, YM30 filters) to a final volume of 5 mls, and 0.5 ml of the concentrated AS supernatant was saved for protein and hGC enzyme activity analysis. The hGC:FLAG™ in 1 ml of concentrated supernatant was purified by affinity chromatography using an anti-FLAG™ affinity column.

To utilize the FLAG™ epitope for purification of the hGC:FLAG™ protein, 1 ml of leaf extract prepared as above was applied to a 1 ml anti-FLAG™ M2 affinity column. The column was previously equilibrated with phosphate-buffered saline (PBS; 50 mM, pH 6.4) containing 10% glycerol and 4 mM β-mercapto-ethanol at 4° C. After several washes with PBS, the bound hGC:FLAG™ protein was eluted with three 1 ml aliquots of purified FLAG™ peptide (IBI), i.e., 1 ml at 500 μg/ml, followed by 2x1 ml at 250 μg/ml. Eluted material was slot-blotted onto a nitrocellulose membrane and tested for immuno-reactivity to the anti-FLAG™ M2 antibody, and analyzed by SDS-PAGE, and stained with Commassie blue to determine relative purity (FIG. 7A). No immuno-reactive material was eluted in the first fraction since release of the bound hGC:FLAG™ protein requires equilibration with the peptide. As a consequence, the second and third eluted fractions contained the majority of immuno-reactive material. SDS-PAGE analysis of anti-FLAG™-purified hGC:FLAG™ protein showed a single band co-migrating with the anti-FLAG™ immuno-reactive protein (FIG. 7A).

In order to utilize the properties of the glycans present on the hGC:FLAG™ protein for purification purposes, hGC:FLAG™ protein was also isolated using a concanavalin-A (ConA) affinity column (Sigma). Concentrated tissue extract (1.5 ml) was loaded onto a 1.5 ml bed volume of ConA in column buffer (0.1M sodium citrate pH 6.5, 0.15M sodium chloride). An equal volume of column buffer was added to the concentrated extract and passed through the column twice at 4° C. The ConA column was washed three times with column buffer using three times the bed volume of buffer. The bound hGC:FLAG™ was eluted with 5 mls of 0.1M methyl α-D-mannopyranoside (Sigma) followed by 5 mls of 1M methyl α-D-mannopyranoside. Fractions were

collected and assayed for protein content and hGC enzymatic activity. All fractions containing hGC enzyme activity were concentrated (Amicon, YM30 filters) to a final volume of 0.5 ml. To stabilize the hGC enzymatic activty of the hGC:FLAG™ protein, the concentrated extract was made 40% (v/v) in glycerol and stored at 4° C. SDS-PAGE analysis of the ConA purified hGC:FLAG™ protein (FIG. 7B) showed a band migrating at 66–69 kd and three lower molecular weight bands that stained equally with Commassie blue.

Enzyme activity and protein determination of fractions from each step in the purification indicate that the most effective method to purify hGC:FLAGm was to employ anti-FLAG™ affinity chromatography followed by the ConA affinity chromatography (see Table 2 and FIGS. 7A–B).

TABLE 2

PURIFICATION OF hGC:FLAG ™ FROM TOBACCO EXTRACTS

| Fraction | Protein Conc. | Specific activity (nmole 4 MU/min/$\mu$g/ml) | % Activity Recovered | Fold Purification |
|---|---|---|---|---|
| 40 gms FW | 2 mg/ml | *0.027 | 100 | 1 |
| 33% AS-sup | 2.5 mg/ml | *0.625 | 180 | 13 |
| ConA | 0.1 mg/ml | +0.81 | 12.5 | 240 |
| FLAG | 7.2 $\mu$g/ml | +0.84 | N.D. | N.D. |

*Since 4 MUGlc is not a specific substrate, this specific activity represents both plant glucosidase and hGC activity.
+ Plant glucosidase does not bind to ConA or anti-FLAG ™ affinity columns (data not shown), therefore, this enzymatic activity is from hGC:FLAG ™ alone.

## 6.4. PRODUCTION OF hGC:FLAG™ PROTEIN FROM TOBACCO PLANTS

An estimation can be made on the amount of hGC: FLAG™ extracted per gm fresh weight of tobacco plant tissue or per mg soluble protein from slot blot western analysis of initial crude extracts using anti-FLAG™. Approximately 2 mg/ml of soluble protein were extracted per 0.5 gm of fresh weight plant tissue. Western slot blot analysis of 1 $\mu$l of crude extract indicates the presence of approximately 0.5 to 0.6 $\mu$g of hGC:FLAG™ (FIG. 8). Based on these results, a single mature tobacco plant comprising about 1.6 kg of fresh weight of tissue will contain about 2.5 gm of hGC:FLAG™ per plant. Accordingly, a standard acre of tobacco planted to 6,000 plants could potentially produce 15 kg of hGC:FLAG™ (Table 3).

TABLE 3

EXTRACTABLE hGC:FLAG ™ PER ACRE OF TOBACCO

| Tissue | Soluble Protein Total | Extractable hGC:FLAG ™ |
|---|---|---|
| *1 gm | 4–5 mg | 1.5 mg |
| 1.6 kg/plant | 6–8 gm | 2.4 gm |
| 6,000 PLANTS/ACRE (Standard field) | | |
| 9,600 kg | 38–48 kg | 14.4 kg |

*These estimations are based on slot blot westerns using anti-FLAG and crude extracts from 0.5 gm–50 gm of post-wounded tissue.

# 7. EXAMPLE 2

## PRODUCTION AND PURIFICATION OF IDUA IN TRANSGENIC TOBACCO PLANTS

The subsections below describe the production of enzymatically active recombinant human α-L-iduronidase (IDUA) in transgenic tobacco plants.

## 7.1. CONSTRUCTION OF A PLANT TRANSFORMATION VECTOR CONTAINING AN IDUA EXPRESSION CONSTRUCT

### 7.1.1. IDUA EXPRESSION CONSTRUCT

The first step in the construction of the desired plant transformation vector was to generate the human IDUA coding region with appropriate flanking restriction site to facilitate fusion to specific plant promoters and insertion into plant transformation vectors. A full-length human IDUA cDNA clone was provided by E. Neufeld (University of California, Los Angeles). In this clone, the IDUA cDNA sequence was inserted into the EcoRI site of pBS plasmid (Moskowitz et al., 1992, FASEB J. 6:A77; Murray, 1987, Methods in Enzymol. 149:25–42). This IDUA cDNA sequence has been expressed in animal cell lines (Moskowitz et al., 1992, supra, 1987, supra) and shown to contained all the information necessary to produce enzymatically active IDUA (Murray, 1987, supra). The IDUA cDNA encodes a 653 amino acid protein (66 kDa) including the 26 amino-terminal signal peptide which is cleaved as it passes through the ER membrane. To aid in the insertion of the IDUA cDNA into the plant vector, unique flanking XbaI and SacI sites were introduced by PCR using 5'-primer ID1 and 3'-primer ID2, Pfu polymerase (Stratagene, La Jolla, Calif.); as shown in FIG. 12. The 1.9 kb fragment generated by PCR was purified by agarose gel electrophoresis, digested with XbaI and SacI, and ligated into pBS and pSP64polyA (Gibco, a vector for in vitro transcription/translation). The PCR-amplified IDUA coding sequence was sequenced prior to insertion into the expression constructs. The nucleotide and deduced amino acid sequences of the amplified IDUA coding sequence are shown in FIGS. 19 (SEQ ID NO:8) and 20 (SEQ ID NO:9), respectively. The PCR-amplified IDUA coding sequence differs from that originally published by E. Neufeld at positions 931 and 932. The PCR-amplified IDUA sequence has the dinucleotide CG instead of the original GC at those positions. Accordingly, the deduced amino acid sequence of the PCR-amplified IDUA has a glutamate, instead of a glutamine, residue at position 282. In vitro transcription of the PCR-amplified IDUA sequence in a pSP64polyA:IDUA vector and rabbit reticulocyte lysate-mediated in vitro translation of the resultant transcript produced protein having a molecular size expected for IDUA.

The PCR-amplified IDUA coding region was inserted downstream of two distinctly regulated plant promoters: 1) the MeGA promoter and 2) the $35S^{ENH}$ promoter. As discussed above, the MeGA promoter shows little or no expression in most plant tissues but is strongly inducible resulting in significant transgene product accumulation 12 to 48 hours after induction of the MeGA promoter. The $35S^{ENH}$ promoter is a widely used high-level constitutive promoter consisting of a modified CaMV 35S promoter containing double enhancer which is fused to a translational enhancer from the tobacco etch virus. See Cramer et al., 1996, "High-Level of Enzymatically Active Human Lysosomal Proteins in Transgenic Tobacco", *Transgenic Plants: A production System for Industrial and Pharmaceutical Proteins*, eds., Owens & Pen, John Wiley & Sons; Chrispeels, 1991, Annu. Rev. Plant Physiol. Plan. Biol. 42:21–53; and Haskins et al., 1979, Pediat. Res. 13:1294–1297. Each promoter was ligated as a HindIII-XbaI fragment upstream of the IDUA cDNA (see FIG. 12).

### 7.1.2. IDUA EXPRESSION/TRANSFORMATION VECTORS

During the subcloning and vector analysis steps, bacterial transformants having any vector containing the 5'-end of the

IDUA cDNA were recovered at lower than expected frequencies. For example, multiple ligation and transformations of competent *E. coli* cells DH5α with pBs containing the 1.9 kb PCR amplified IDUA cDNA were required to generate fewer than 100 transformants. Among the 70 transformants analyzed by restriction analysis of the plasmid DNA, only 2 clones contained the proper sized 1.9 kb fragment. One of the two clones was sequenced and found to contain the complete IDUA coding sequence. Colony size of IDUA containing transformant was reduced. These reduced efficiencies were independent of plasmid vector, presence or absence of plant promoter, IDUA expression (not fused to a bacterially active promoter) or bacterial host. Independent subcloning of the 3'-versus 5'-end of the IDUA cDNA localized an "obnoxious" region to the 5'-end of the IDUA sequence. DNA secondary structure or the high GC content of this region may cause intolerance in heterologous organisms. This effect by the 5'-end of the IDUA cDNA has also been noticed in yeast and animal cell expression systems. These limitations in transformation of the IDUA sequence, however, did not preclude successful isolation and characterization of the desired IDUA expression and transformation constructs.

For both promoter constructs, the promoter:IDUA cDNA fusions were excised as HindIII/SacI fragments and ligated into HindIII and SacI-digested pBIB-KAN (FIG. 12). pBIB-Kan is a large (>13 kb) plant transformation vector that provides a terminator/polyadenylation signal (pAnos) for the introduced transgene, a selectable marker (NPTII or kzinamycin resistance) for transformed plant cells, and T-DNA border sequences that demarcate the DNA to be transferred (Becker, 1990, Nucl. Acids Res. 18:203). The recombinant vectors were propagated in *E. coli* and fully characterized prior to transfer to *Agrobacterium tumefaciens*. A pBIB-KAN vector containing the MeGA:IDUA expression construct used in T-DNA transformation of plants is pCT22.

7.2. GENERATION OF TRANSGENIC TOBACCO CONTAINING THE IDUA CONSTRUCTS

Agrobacterium-mediated transformation was used to stably integrate the $35S^{ENH}$:IDUA and MeGA:IDUA constructs into the genome of tobacco. Approximately 80 leaf discs were excised from aseptically grown *Nicotiana tabacum* cvs. *Xanthi* seedlings for each gene construct and inoculated with suspension cultures of *A. tumefaciens* strains containing the IDUA expression/transformation vectors. Following a 48 hour co-cultivation period, the leaf discs were transferred to selection media containing kanamycin and hormones that promote shoot formation. Although numerous shoots (4–10 per disc) generally appear 2–3 weeks after transfer to selection media, the IDUA-transformed shoots appeared late, i.e., after 3–5 weeks, and were few in number (0–1 per disc). Induction of root formation was also delayed in the IDUA-transformed shoots compared to shoots containing other transgene constructs. A final yield of seven $35S^{ENH}$:IDUA and ten MeGA:IDUA plantlets were transferred to soil. Once in soil, all plants grew to maturity with normal morphology, flowering, and seed production. IDUA-expressing progenies showed slight retardation in early growth (FIG. 13B) but were indistinguishable in size and appearance from untransformed plants at full maturity.

7.3. SOUTHERN CHARACTERIZATION OF TRANSGENIC PLANTS

Transgenic plants were initially selected based on kanamycin resistance. The stable insertion of the MeGA:IDUA gene construct was confirmed by genomic Southern hybridization analysis. Total DNA was isolated from leaf tissue of nine transgenic plants and digested with HindIII, and analyzed by Southern hybridization using the IDUA cDNA as probe. The nine putative transformants analyzed showed one to three copies of the IDUA insert and no indication of rearrangements or deletions. This transgene copy number is typical of transgenic tobacco engineered with other constructs via Agrobacterium.

7.4. CHARACTERIZATION OF IDUA EXPRESSION IN TRANSGENIC PLANTS

7.4.1. IMMUNO-DETECTION OF IDUA PROTEIN IN PLANT EXTRACT

Antibodies made to the native and denatured IDUA from CHO cells were obtained from E. Kakkis (Harbor-UCLA Medical Center, Los Angeles, Calif.). By immuno-slot blot and SDS-PAGE Western analysis, the antibodies were found not to react with any proteins in untransformed or pBIB-Kan (transformed vector alone) transgenic tobacco tissue extracts from uninduced or induced leaf tissue. When purified IDUA from CHO cells was seeded to untransformed tobacco extracts, there was no diminution in the level of IDUA detected as compared to that detected in extraction buffer containing the same concentration of purified IDUA. This finding indicates that tobacco extract does not inhibit immuno-detection of IDUA.

Leaf tissues from seven independent transgenic plants were harvested, homogenized in 3X volume of extraction buffer (PBS with 0.1% Triton X100, 200 μM PMSF, 1 μM pepstatin, 4 μM leupeptin) and the extracts cleared of cell debris by centrifugation at 12,000×g for 30 min. Twenty-five μg of total soluble protein from each extract was heat-denatured and slotted onto OPTITRAN membrane (S&S). Purified IDUA protein in amounts ranging from 20 ng to 400 ng were added to the membrane to serve as comparison standards. Based on antibody detection using chemiluminescence, no immuno-reactive IDUA protein was found in the extracts of any of the $35S^{ENH}$:IDUA transgenic plants. This constitutive promoter also poorly expressed human protein C (<0.02% of soluble protein). Based on these findings, the $35S^{ENH}$:IDUA-containing plants were not analyzed further.

The MeGA promoter is inactive in tobacco leaves in the absence of induction. To obtain IDUA expression, leaves were harvested, induced by mechanical wounding and incubated at room temperature under high humidity (i.e., the wounded leaves are wrapped in moist filter paper in sealed bags or layered in a container with buffer gently swirled over the tissue) to allow de novo synthesis of the transgene product. In an initial screen of ten MeGA:IDUA-containing plants, tissue extracts were used for immunodot-blot analyses (see above). The extracts showed little or no IDUA content for all plants. Later analyses revealed that IDUA was secreted from the leaves and leached out onto the filter paper during the incubation step. This was somewhat surprising because recovery of extracellular proteins from intact leaf generally requires vacuum-induced buffer infiltration of the leaf (see Parent & Asselin, 1987, Can. J. Bot. 62:564–569; Regalado & Ricardo, 1996, Plant Physiol. 110:227–232). As described below, the expression procedure was subsequently modified to include a post-induction incubation step that involved gentle rotation of buffer over the wounded tissue, which permitted recovery of IDUA protein and activity in the incubation buffer. Subsequent analyses were focused primarily on one plant, IDUA-9 also known as CT40-9, since preliminary tests show detectable levels of IDUA activity and anti-IDUA immuno-reactive material. IDUA-9 contains 3 copies of the MeGA:IDUA construct.

7.4.2. NORTHERN ANALYSIS SHOWS ACTIVATION OF THE MEGA:IDUA TRANSGENE

In order to demonstrate induction of the MeGA promoter and accumulation of IDUA mRNA, total RNA was isolated (Rutter, 1981, J. Biol. Chem. 91:468–478) from IDUA-9 leaves before and after induction. As shown in FIG. 14A, IDUA mRNA of the expected size (approximately 2.2 kb) was detected at low basal levels in uninduced tissue and showed a marked increase at 8 hrs post-induction and reached a maximum level at 27 hrs post-induction. This pattern is similar to transgene induction kinetics seen with other MeGA-driven constructs (e.g., hGC:FLAG™). The smaller hybridizing RNA species also accumulated after induction. Analogous lower molecular weight RNAs have not been detected in hGC:FLAG™ expressing plants and may be unique to the IDUA-9 plant or a consequence of the IDUA sequence.

7.4.3. WESTERN ANALYSIS OF HUMAN IDUA LOCALIZED TO TOBACCO

The induced IDUA-9 tissues were also used for protein extracts. Western blot analysis showed CHO-derived IDUA and IDUA from tobacco tissue migrated very similarly in SDS-PAGE (FIG. 14B). The IDUA (92 kD) from IDUA-9 tobacco extract migrated slightly faster than secreted IDUA from CHO cells. This presumably is due to differences in glycan composition. However, the similarity in size suggests that the tobacco produced recombinant IDUA was also glycosyleated.

7.4.4. IDUA SYNTHESIZED IN TRANSGENIC TOBACCO IS SECRETED

As discussed above, CHO cells secrete recombinant IDUA into the media. To determine if tobacco also secrete recombinant IDUA into the media, leaf tissue from transgenic IDUA-7, -8 and -9 plants were induced for 0 to 34 hrs and placed in a plastic petri dish with incubation buffer (PBS). At 0 hr, incubation buffer was used to wash the induced tissue and the wash stored frozen. Fresh buffer was added to the induced tissue and incubated at room temperature. At 8 hrs, the buffer was removed and frozen. Fresh buffer was added to the induced tissue and incubated further. The buffer was removed at 24 hrs post-induction. Fresh buffer was added to the induced tissue and further incubated. The final incubation buffer was removed 34 hrs post-induction and a tissue extract was prepared from the incubated leaf tissue. Fifty μl of each incubation buffer and tissue extract was boiled and slotted onto OPTITRAN membrane. A range of control IDUA protein from 0 to 40 ng was also blotted and IDUA was detected using anti-IDUA antibodies. As shown in FIG. 15, IDUA protein was present in the incubation buffer following induction in all three transgenic tissue analyzed. This indicates that transgenic tobacco secrete IDUA after synthesis.

7.4.5. THE TOBACCO-SYNTHESIZED IDUA IS ENZYMATICALLY ACTIVE

One of the most critical factors in assessing the utility of plant-synthesized recombinant IDUA is whether the IDUA is enzymatically active. Enzyme activity of human lysosomal hydrolases requires appropriate glycosylation and folding and heterologous expression systems often result in endoplasmic reticulum-localized degradation or accumulation of insoluble and inactive aggregates. To determine whether the recombinant IDUA synthesized in transgenic leaves has enzymatic activity, a sensitive fluorometric assay using the substrate, 4-Methylumbelliferyl-α-L-iduronide (4-MUI) (Calbiochem, LaJolla, Calif.) was used (see Neufeld, E. F., 1991, Ann. Rev. Biochem. 60:257–280). Untransformed tobacco extracts were shown to contain no endogenous IDUA activity. When CHO-derived recombinant IDUA was seeded into crude extracts of untransformed tobacco leaves, no detectable inhibition of activity was found. When the tissue extracts from IDUA-9 transgenic plant were assayed, the extracts showed IDUA activity at reproducible but at relatively low levels (0.2 to 0.4 nmole 4-MU/hr/gm tissue). This confirms that tobacco has all the necessary machinery to synthesize and process IDUA into an active form. Consistent with IDUA distribution shown by immuno-detection, significantly higher IDUA activities were detected in the secreted fraction as described below.

7.4.6. SECRETION AND RECOVERY OF TOBACCO-SYNTHESIZED RECOMBINANT IDUA

Significant portion of the recombinant IDUA produced in transgenic tobacco was recovered in the incubation buffer following induction of the MeGA:IDUA gene construct (FIG. 15). Localization of the majority of active IDUA after induction and incubation was determined. This was done by comparing the IDUA activity and anti-IDUA immunoreactivity of tissue extract with those of the incubation buffer. As shown in FIG. 16, there was much higher levels of IDUA activity in the incubation buffer than in the tissue extract after induction and incubation. Moreover, the IDUA activity in the incubation buffer showed strong correlation with the the amount of anti-IDUA immuno-reactive material found in the incubation buffer, as reveal by the data presented in FIG. 15. Thus, IDUA-expressing transgenic tobacco secrete most of its active IDUA (about 67%) into the incubation buffer after induction and incubation.

Based on activity assays and Western analysis, the specific activity of secreted IDUA was estimated to be about 64 U/μg protein. In comparison, purified IDUA enzyme from engineered CHO cells has a specific activity of about 242 U/μg protein.

Variation in transgene expression levels is very common in transgenic plants due to "positional" effects caused by the site of transgene insertion within the host genome. The IDUA activity levels in three independent IDUA-expressing transgenic plants (i.e., IDUA-7, IDUA-8 and IDUA-9) were examined. Among these transgenic plants, IDUA-9 has the highest IDUA activity (FIG. 17). The relative amount of active IDUA remaining in the cell, as reflected by the activity present in tissue extract, after 34 hrs of incubation ranged from 14% to 35% of the total activity (FIG. 17).

The above-identified three transgenic plants were identified in a screen of about fifty independently transformed plants. This is a relatively small scale screen. It is reasonable to expect that larger scale Ecreenings of IDUA-engineered plants will yield plants that produce active IDUA at levels higher than those of the plantes disclosed herein.

7.4.7. PURIFICATION AND YIELD OF IDUA FROM TRANSGENIC TOBACCO

The yield of recombinant IDUA from IDUA-9 was estimated to be about 6 μg/gm fresh tissue. This estimate was based on the material present in the incubation buffer after 34 hrs of incubation (see FIG. 18). However, neither the induction nor the IDUA recovery procedure used was optimized. Thus, it is likely that higher IDUA yields may be acheived through optimization of induction and recovery procedures. It should be noted that the transgenic tobacco plants yielded an average of greater than 1 kg fresh weight of leaf at maturity, and that leaves can be periodically harvested from greenhouse-grown plants for over an year. Accordingly, cultivation of transgenic tobacco plants either in the field of the greenhouse offers a convenient and effective means for producing large amounts of IDUA.

8. EXAMPLE 3

PRODUCTION OF TRANSGENIC TOBACCO PLANTS CONTAINING AN UNMODIFIED hGC EXPRESSION CONSTRUCT

A 3' end segment of the hGC coding sequence was PCR-amplified from the cDNA clone in *E. coli* ATCC65696

(see Section 6.1.1., supra) using as the 5' primer GC23 oligo, 5'GCCTATGCTGAGCACAAGTTACAG3' (SEQ ID NO:11), whose 5' end corresponds to nucleotide 894 of the hGC:FLAG sequence shown in FIG. 9, and as the 3' primer GC37 oligo, whose complementary strand has the sequence 5' TTCCTTGAGCTCGtcaCTGGCGACGCCACAGGTA3' (SEQ ID NO:12), a SacI restriction site is shown with an underline and a stop codon that is in-frame to the amplified hGC coding sequence is shown in lower case. The site of the 5' primer in the hGC coding sequence is 5' upstream of a SalI restriction site. Accordingly, the amplified DNA was cut with SalI and SacI, and the SalI/SacI fragment containing the 3' end of hGC coding sequence was inserted into the pBS intermediate vector containing the MeGA:hGC:FLAG™ expression construct (see FIG. 1 and Section 6.1.2., supra) which had been cut with SalI and SacI. Clones were identified that had replaced the 3' end of the MeGA:hGC-:FLAG™ construct with the 3' end of hGC coding sequence yielding a MeGA:hGC expression construct. This construction eliminated the ten amino acid addition at the carboxyl terminal and corrected the amino acid substitution at residue 545 in the hGC:FLAG™ fusion, and thereby reconstructing an unmodified hGC coding sequenze. The MeGA:hGC expression construct was excised from the pBS intermediate vector by SacI digestion and inserted into pBIB-KAN to form the transformation vector pCT54. Ak schematic of the construction of the pCT54 vector is shown in FIG. 21.

Agrobacterium containing pCT54 was used to tranformed plants and transgenic tobacco plants containing the MeGA-:hGC expression construct were produced according to procedures described aboveTransgenic tobacco plants containing the MeGA:hGC expression construct were identified atnd assigned the designations CT54-1 to -40. Analyses of hGC enzymatic activity and presence of hGC in the induced tissues of transgenic plants are carried out using the enzymatic assay described in Section 6.2.5. and the Western blot analysis using anti-hGC antibodies described in Section 6.2.6. Purification of the hGC produced in transgenic tobacco tissue is carried out using the procedure described in



Section 6.3., except the anti-FLAG™ affinity chromatography step was omitted, which procedure is further modified accordingly to strategies and methods known in the art for purifying the hGC enzyme.

## 9. DEPOSIT OF BIOLOGICAL MATERIALS

The following biological materials have been deposited with the American Type Culture Collection (ATCC) at 12301 Parklawn Drive, Rockville, Md. 20852, in compliance with the requirements of the Budapest Treaty On The International Recognition Of The Deposit Of Microorganisms For The Purpose Of Patent Procedure, on the dates and were assigned the ATCC accession numbers indicated below.

| Deposited Material | Deposit Date | Accession No. |
|---|---|---|
| DNA of pCTPro1:hGC:FLAG | Sept. 14, 1995 | 97277 |
| seeds of tobacco plant hGC X-11 | Sept. 14, 1995 | 97275 |
| seeds of tobacco plant hGC X-27 | Sept. 14, 1995 | 97276 |
| DNA of pCT22 | Aug. 30, 1996 | 97701 |
| seeds of tobacco plant CT40-9 | Aug. 30, 1996 | 97700 |
| DNA of pCT54 | Oct. 17, 1996 | 97770 |

The present invention is not to be limited in scope by the biological material deposited since the deposited embodiments are intended as illustrations of the individual aspects of the invention, and any biological material, or constructs which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the airt from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein; these are incorporated by reference in their entirety.

SEQUENCE LISTING

```
(1) GENERAL INFORMATION:

   (iii) NUMBER OF SEQUENCES: 15

(2) INFORMATION FOR SEQ ID NO:1:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 27 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: other nucleic acid
          (A) DESCRIPTION: /desc = "PCR primer"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTGTCTAGAG TAAGCATCAT GGCTGGC                                27

(2) INFORMATION FOR SEQ ID NO:2:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 33 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
```

-continued

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CACGAATTCT GGCGACGCCA CAGGTAGGTG TGA 33

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1642 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGGAGTTTT CAAGTCCTTC CAGAGAGGAA TGTCCCAAGC CTTTGAGTAG GGTAAGCATC 60

ATGGCTGGCA GCCTCACAGG TTTGCTTCTA CTTCAGGCAG TGTCGTGGGC ATCAGGTGCC 120

CGCCCCTGCA TCCCTAAAAG CTTCGGCTAC AGCTCGGTGG TGTGTGTCTG CAATGCCACA 180

TACTGTGACT CCTTTGACCC CCCGACCTTT CCTGCCCTTG GTACCTTCAG CCGCTATGAG 240

AGTACACGCA GTGGGCGACG GATGGGGCTG AGTATGGGGC CCATCCAGGC TAATCACACG 300

GGCACAGGCC TGCTACTGAC CCTGCAGCCA GAACAGAAGT TCCAGAAAGT GAAGGGATTT 360

GGAGGGGCCA TGACAGATGC TGCTGCTCTC AACATCCTTG CCCTGTCACC CCCTGCCCAA 420

AATTTGCTAC TTAAATCGTA CTTCTCTGAA GAAGGAATCG GATATAACAT CATCCGGGTA 480

CCCATGGCCA GCTGTGACTT CTCCATCCGC ACCTACACCT ATGCAGACAC CCCTGATGAT 540

TTCCAGTTGC ACAACTTCAG CCTCCCAGAG GAAGATACCA AGCTCAAGAT ACCCCTGATT 600

CACCGAGCCC TGCAGTTGGC CCAGCGTCCC GTTTCACTCC TTGCCAGCCC CTGGACATCA 660

CCCACTTGGC TCAAGACCAA TGGAGCGGTG AATGGGAAGG GGTCACTCAA GGGACAGCCC 720

GGAGACATCT ACCACCAGAC CTGGGCCAGA TACTTTGTGA AGTTCCTGGA TGCCTATGCT 780

GAGCACAAGT TACAGTTCTG GGCAGTGACA GCTGAAAATG AGCCTTCTGC TGGGCTGTTG 840

AGTGGATACC CCTTCCAGTG CCTGGGCTTC ACCCCTGAAC ATCAGCGAGA CTTCATTGCC 900

CGTGACCTAG GTCCTACCCT CGCCAACAGT ACTCACCACA ATGTCCGCCT ACTCATGCTG 960

GATGACCAAC GCTTGCTGCT GCCCCACTGG GCAAAGGTGG TACTGACAGA CCCAGAAGCA 1020

GCTAAATATG TTCATGGCAT TGCTGTACAT TGGTACCTGG ACTTTCTGGC TCCAGCCAAA 1080

GCCACCCTAG GGGAGACACA CCGCCTGTTC CCCAACACCA TGCTCTTTGC CTCAGAGGCC 1140

TGTGTGGGCT CCAAGTTCTG GGAGCAGAGT GTGCGGCTAG GCTCCTGGGA TCGAGGGATG 1200

CAGTACAGCC ACAGCATCAT CACGAACCTC CTGTACCATG TGGTCGGCTG GACCGACTGG 1260

AACCTTGCCC TGAACCCCGA AGGAGGACCC AATTGGGTGC GTAACTTTGT CGACAGTCCC 1320

ATCATTGTAG ACGTCACCAG GGACACGTTT TACAAACAGC CCATGTTCTA CCACCTTGGC 1380

CACTTCAGCA AGTTCATTCC TGAGGGCTCC CAGAGAGTGG GGCTGGTTGC CAGTCAGAAG 1440

AACGACCTGG ACGCAGTGGC ACTGATGCAT CCCGATGGCT CTGCTGTTGT GGTCGTGCTA 1500

AACCGCTCCT CTAAGGATGT GCCTCTTACC ATCAAGGATC CTGCTGTGGG CTTCCTGGAG 1560

ACAATCTCAC CTGGCTACTC CATTCACACC TACCTGTGGC GTCGCCAGAA TTCGGACTAC 1620

AAGGACGACG ATGACAAGTT GA 1642

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 546 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Glu Phe Ser Ser Pro Ser Arg Glu Glu Cys Pro Lys Pro Leu Ser
1               5                   10                  15

Arg Val Ser Ile Met Ala Gly Ser Leu Thr Gly Leu Leu Leu Leu Gln
            20                  25                  30

Ala Val Ser Trp Ala Ser Gly Ala Arg Pro Cys Ile Pro Lys Ser Phe
        35                  40                  45

Gly Tyr Ser Ser Val Val Cys Val Cys Asn Ala Thr Tyr Cys Asp Ser
    50                  55                  60

Phe Asp Pro Pro Thr Phe Pro Ala Leu Gly Thr Phe Ser Arg Tyr Glu
65                  70                  75                  80

Ser Thr Arg Ser Gly Arg Arg Met Glu Leu Ser Met Gly Pro Ile Gln
                85                  90                  95

Ala Asn His Thr Gly Thr Gly Leu Leu Leu Thr Leu Gln Pro Glu Gln
            100                 105                 110

Lys Phe Gln Lys Val Lys Gly Phe Gly Gly Ala Met Thr Asp Ala Ala
        115                 120                 125

Ala Leu Asn Ile Leu Ala Leu Ser Pro Pro Ala Gln Asn Leu Leu Leu
    130                 135                 140

Lys Ser Tyr Phe Ser Glu Glu Gly Ile Gly Tyr Asn Ile Ile Arg Val
145                 150                 155                 160

Pro Met Ala Ser Cys Asp Phe Ser Ile Arg Thr Tyr Thr Tyr Ala Asp
                165                 170                 175

Thr Pro Asp Asp Phe Gln Leu His Asn Phe Ser Leu Pro Glu Glu Asp
            180                 185                 190

Thr Lys Leu Lys Ile Pro Leu Ile His Arg Ala Leu Gln Leu Ala Gln
        195                 200                 205

Arg Pro Val Ser Leu Leu Ala Ser Pro Trp Thr Ser Pro Thr Trp Leu
    210                 215                 220

Lys Thr Asn Gly Ala Val Asn Gly Lys Gly Ser Leu Lys Gly Gln Pro
225                 230                 235                 240

Gly Asp Ile Tyr His Gln Thr Trp Ala Arg Tyr Phe Val Lys Phe Leu
                245                 250                 255

Asp Ala Tyr Ala Glu His Lys Leu Gln Phe Trp Ala Val Thr Ala Glu
            260                 265                 270

Asn Glu Pro Ser Ala Gly Leu Leu Ser Gly Tyr Pro Phe Gln Cys Leu
        275                 280                 285

Gly Phe Thr Pro Glu His Gln Arg Asp Phe Ile Ala Arg Asp Leu Gly
    290                 295                 300

Pro Thr Leu Ala Asn Ser Thr His His Asn Val Arg Leu Leu Met Leu
305                 310                 315                 320

Asp Asp Gln Arg Leu Leu Leu Pro His Trp Ala Lys Val Val Leu Thr
                325                 330                 335

Asp Pro Glu Ala Ala Lys Tyr Val His Gly Ile Ala Val His Trp Tyr
            340                 345                 350

Leu Asp Phe Leu Ala Pro Ala Lys Ala Thr Leu Gly Glu Thr His Arg
        355                 360                 365
```

-continued

Leu Phe Pro Asn Thr Met Leu Phe Ala Ser Glu Ala Cys Val Gly Ser
    370                 375                 380

Lys Phe Trp Glu Gln Ser Val Arg Leu Gly Ser Trp Asp Arg Gly Met
385                 390                 395                 400

Gln Tyr Ser His Ser Ile Ile Thr Asn Leu Leu Tyr His Val Val Gly
                405                 410                 415

Trp Thr Asp Trp Asn Leu Ala Leu Asn Pro Glu Gly Gly Pro Asn Trp
            420                 425                 430

Val Arg Asn Phe Val Asp Ser Pro Ile Ile Val Asp Val Thr Lys Asp
        435                 440                 445

Thr Phe Tyr Lys Gln Pro Met Phe Tyr His Leu Gly His Phe Ser Lys
    450                 455                 460

Phe Ile Pro Glu Gly Ser Gln Arg Val Gly Leu Val Ala Ser Gln Lys
465                 470                 475                 480

Asn Asp Leu Asp Ala Val Ala Leu Met His Pro Asp Gly Ser Ala Val
                485                 490                 495

Val Val Val Leu Asn Arg Ser Ser Lys Asp Val Pro Leu Thr Ile Lys
            500                 505                 510

Asp Pro Ala Val Gly Phe Leu Glu Thr Ile Ser Pro Gly Tyr Ser Ile
        515                 520                 525

His Thr Tyr Leu Trp Arg Arg Gln Asn Ser Asp Tyr Lys Asp Asp Asp
    530                 535                 540

Asp Lys
545

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 471 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "MeGA Promoter"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAATACGATA TTACCGAATA TTATACTAAA TCAAAATTTA ATTTATCATA TCGAATTATT 60

AAACTGATAT TTCAAATTTT AATATTTAAT ATCTACTTTC AACTATTATT ACCTAATTAT 120

CAAATGCAAA ATGTATGAGT TATTTCATAA TAGCCCGAGT TCGTATCCAA ATATTTTACA 180

CTTGACCAGT CAACTTGACT ATATAAAACT TTACTTCAAA AAATTAAAAA AAAAAGAAAG 240

TATATTATTG TAAAAGATAA TACTCCATTC AAAATATAAA ATGAAAAAAG TCCAGCGCGG 300

CAACCGGGTT CCTCTATAAA TACATTTCCT ACATCTTCTC TTCTCCTCAC ATCCCATCAC 360

TCTTCTTTTA ACAATTATAC TTGTCAATCA TCAATCCCAC AAACAACACT TTTTCTCTCC 420

TCTTTTTCCT CACCGGCGGC AGACTTACCG GTGAAATCTA GAGTAAGCAT C 471

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

-continued

CTAGTCTAGA ATGCGTCCCC TGCGCCCCCG CG 32

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGAATTCGAG CTCTCATGGA TTGCCCGGGG ATG 33

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2067 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGCGTCCCC TGCGCCCCCG CGCCGCGCTG CTGGCGCTCC TGGCCTCGCT CCTGGCCGCG 60

CCCCCGGTGG CCCCGGCCGA GGCCCCGCAC CTGGTGCAGG TGGACGCGGC CCGCGCGCTG 120

TGGCCCCTGC GGCGCTTCTG GAGGAGCACA GGCTTCTGCC CCCCGCTGCC ACACAGCCAG 180

GCTGACCAGT ACGTCCTCAG CTGGGACCAG CAGCTCAACC TCGCCTATGT GGGCGCCGTC 240

CCTCACCGCG GCATCAAGCA GGTCCGGACC CACTGGCTGC TGGAGCTTGT CACCACCAGG 300

GGGTCCACTG GACGGGGCCT GAGCTACAAC TTCACCCACC TGGACGGGTA CTTGGACCTT 360

CTCAGGGAGA ACCAGCTCCT CCCAGGGTTT GAGCTGATGG GCAGCGCCTC GGGCCACTTC 420

ACTGACTTTG AGGACAAGCA GCAGGTGTTT GAGTGGAAGG ACTTGGTCTC CAGCCTGGCC 480

AGGAGATACA TCGGTAGGTA CGGACTGGCG CATGTTTCCA AGTGGAACTT CGAGACGTGG 540

AATGAGCCAG ACCACCACGA CTTTGACAAC GTCTCCATGA CCATGCAAGG CTTCCTGAAC 600

TACTACGATG CCTGCTCGGA GGGTCTGCGC GCCGCCAGCC CCGCCCTGCG GCTGGGAGGC 660

CCCGGCGACT CCTTCCACAC CCCACCGCGA TCCCCGCTGA GCTGGGGCCT CCTGCGCCAC 720

TGCCACGACG GTACCAACTT CTTCACTGGG GAGGCGGGCG TGCGGCTGGA CTACATCTCC 780

CTCCACAGGA AGGGTGCGCG CAGCTCCATC TCCATCCTGG AGCAGGAGAA GGTCGTCGCG 840

CACGAGATCC GGCAGCTCTT CCCCAAGTTC GCGGACACCC CCATTTACAA CGACGAGGCG 900

GACCCGCTGG TGGGCTGGTC CCTGCCACAG CCGTGGAGGG CGGACGTGAC CTACGCGGCC 960

ATGGTGGTGA AGGTCATCGC GCAGCATCAG AACCTGCTAC TGGCCAACAC CACCTCCGCC 1020

TTCCCCTACG CGCTCCTGAG CAACGACAAT GCCTTCCTGA GCTACCACCC GCACCCCTTC 1080

GCGCAGCGCA CGCTCACCGC GCGCTTCCAG GTCAACAACA CCCGCCCGCC GCACGTGCAG 1140

CTGTTGCGCA AGCCGGTGCT CACGGCCATG GGGCTGCTGG CGCTGCTGGA TGAGGAGCAG 1200

CTCTGGGCCG AAGTGTCGCA GGCCGGGACC GTCCTGGACA GCAACCACAC GGTGGGCGTC 1260

CTGGCCAGCG CCCACCGCCC CCAGGGCCCG GCCGACGCCT GGCGCGCCGC GGTGCTGATC 1320

TACGCGAGCG ACGACACCCG CGCCCACCCC AACCGCAGCG TCGCGGTGAC CCTGCGGCTG 1380

CGCGGGGTGC CCCCCGGCCC GGGCCTGGTC TACGTCACGC GCTACCTGGA CAACGGGCTC 1440

TGCAGCCCCG ACGGCGAGTG GCGGCGCCTG GGCCGGCCCG TCTTCCCCAC GGCAGAGCAG 1500

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TTCCGGCGCA TGCGCGCGGC TGAGGACCCG GTGGCCGCGG CGCCCCGCCC CTTACCCGCC   1560
GGCGGCCGCC TGACCCTGCG CCCCGCGCTG CGGCTGCCGT CGCTTTTGCT GGTGCACGTG   1620
TGTGCGCGCC CCGAGAAGCC GCCCGGGCAG GTCACGCGGC TCCGCGCCCT GCCCCTGACC   1680
CAAGGGCAGC TGGTTCTGGT CTGGTCGGAT GAACACGTGG GCTCCAAGTG CCTGTGGACA   1740
TACGAGATCC AGTTCTCTCA GGACGGTAAG GCGTACACCC CGGTCAGCAG GAAGCCATCG   1800
ACCTTCAACC TCTTTGTGTT CAGCCCAGAC ACAGGTGCTG TCTCTGGCTC CTACCGAGTT   1860
CGAGCCCTGG ACTACTGGGC CCGACCAGGC CCCTTCTCGG ACCCTGTGCC GTACCTGGAG   1920
GTCCCTGTGC CAAGAGGGCC CCCATCCCCG GGCAATCCAT GAGCCTGTGC TGAGCCCCAG   1980
TGGGTTGCAC CTCCACCGGC AGTCAGCGAG CTGGGGCTGC ACTGTGCCCA TGCTGCCCTC   2040
CCATCACCCC CTTTGCAATA TATTTTT                                       2067
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 653 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Met Arg Pro Leu Arg Pro Arg Ala Ala Leu Leu Ala Leu Leu Ala Ser
1               5                   10                  15

Leu Leu Ala Ala Pro Pro Val Ala Pro Ala Glu Ala Pro His Leu Val
            20                  25                  30

His Val Asp Ala Ala Arg Ala Leu Trp Pro Leu Arg Arg Phe Trp Arg
        35                  40                  45

Ser Thr Gly Phe Cys Pro Pro Leu Pro His Ser Gln Ala Asp Gln Tyr
    50                  55                  60

Val Leu Ser Trp Asp Gln Gln Leu Asn Leu Ala Tyr Val Gly Ala Val
65                  70                  75                  80

Pro His Arg Gly Ile Lys Gln Val Arg Thr His Trp Leu Leu Glu Leu
                85                  90                  95

Val Thr Thr Arg Gly Ser Thr Gly Arg Gly Leu Ser Tyr Asn Phe Thr
            100                 105                 110

His Leu Asp Gly Thr Leu Asp Leu Leu Arg Glu Asn Gln Leu Leu Pro
        115                 120                 125

Gly Phe Glu Leu Met Gly Ser Ala Ser Gly His Phe Thr Asp Phe Glu
    130                 135                 140

Asp Lys Gln Gln Val Phe Glu Trp Lys Asp Leu Val Ser Ser Leu Ala
145                 150                 155                 160

Arg Arg Tyr Ile Gly Arg Tyr Gly Leu Ala His Val Ser Lys Trp Asn
                165                 170                 175

Phe Glu Thr Trp Asn Glu Pro Asp His His Asp Phe Asp Asn Val Ser
            180                 185                 190

Met Thr Met Gln Gly Phe Leu Asn Tyr Tyr Asp Ala Cys Ser Glu Gly
        195                 200                 205

Leu Arg Ala Ala Ser Pro Ala Leu Arg Leu Gly Gly Pro Gly Asp Ser
    210                 215                 220

Phe His Thr Pro Pro Arg Ser Pro Leu Ser Trp Gly Leu Leu Arg His
225                 230                 235                 240

Cys His Asp Gly Thr Asn Phe Phe Thr Gly Glu Ala Gly Val Arg Leu
```

-continued

245 250 255

Asp Tyr Ile Ser Leu His Arg Lys Gly Ala Arg Ser Ser Ile Ser Ile
260 265 270

Leu Glu Gln Glu Lys Val Val Ala Gln Glu Ile Arg Gln Leu Phe Pro
275 280 285

Lys Phe Ala Asp Thr Pro Ile Tyr Asn Asp Glu Ala Asp Pro Leu Val
290 295 300

Gly Trp Ser Leu Pro Gln Pro Trp Arg Ala Asp Val Thr Tyr Ala Ala
305 310 315 320

Met Val Val Lys Val Ile Ala Gln His Gln Asn Leu Leu Leu Ala Asn
325 330 335

Thr Thr Ser Ala Phe Pro Tyr Ala Leu Leu Ser Asn Asp Asn Ala Phe
340 345 350

Leu Ser Tyr His Pro His Pro Phe Ala Gln Arg Thr Leu Thr Ala Arg
355 360 365

Phe Gln Val Asn Asn Thr Arg Pro Pro His Val Gln Leu Leu Arg Lys
370 375 380

Pro Val Leu Thr Ala Met Gly Leu Leu Ala Leu Leu Asp Glu Glu Gln
385 390 395 400

Leu Trp Ala Glu Val Ser Gln Ala Gly Thr Val Leu Asp Ser Asn His
405 410 415

Thr Val Gly Val Leu Ala Ser Ala His Arg Pro Gln Gly Pro Ala Asp
420 425 430

Ala Trp Arg Ala Ala Val Leu Ile Tyr Ala Ser Asp Asp Thr Arg Ala
435 440 445

His Pro Asn Arg Ser Val Ala Val Thr Leu Arg Leu Arg Gly Val Pro
450 455 460

Pro Gly Pro Gly Leu Val Tyr Val Thr Arg Tyr Leu Asp Asn Gly Leu
465 470 475 480

Cys Ser Pro Asp Gly Glu Trp Arg Arg Leu Gly Arg Pro Val Phe Pro
485 490 495

Thr Ala Glu Gln Phe Arg Arg Met Arg Ala Ala Glu Asp Pro Val Ala
500 505 510

Ala Ala Pro Arg Pro Leu Pro Ala Gly Gly Arg Leu Thr Leu Arg Pro
515 520 525

Ala Leu Arg Leu Pro Ser Leu Leu Leu Val His Val Cys Ala Arg Pro
530 535 540

Glu Lys Pro Pro Gly Gln Val Thr Arg Leu Arg Ala Leu Pro Leu Thr
545 550 555 560

Gln Gly Gln Leu Val Leu Val Trp Ser Asp Glu His Val Gly Ser Lys
565 570 575

Cys Leu Trp Thr Tyr Glu Ile Gln Phe Ser Gln Asp Gly Lys Ala Tyr
580 585 590

Thr Pro Val Ser Arg Lys Pro Ser Thr Phe Asn Leu Phe Val Phe Ser
595 600 605

Pro Asp Thr Gly Ala Val Ser Gly Ser Tyr Arg Val Arg Ala Leu Asp
610 615 620

Tyr Trp Ala Arg Pro Gly Pro Phe Ser Asp Pro Val Pro Tyr Leu Glu
625 630 635 640

Val Pro Val Pro Arg Gly Pro Pro Ser Pro Gly Asn Pro
645 650

(2) INFORMATION FOR SEQ ID NO:10:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCTATGCTG AGCACAAGTT ACAG 24

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Complementary sequence of a PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCCCTTGAGC TCGTCACTGG CGACGCCACA GGTA 34

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAGAATTCGG ACTACAAGGA CGACGATGAC AAGTAGGAGC TCGAATTC 48

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Asn Ser Asp Tyr Lys Asp Asp Asp Asp Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

-continued

```
(i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 4 amino acids
     (B) TYPE: amino acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Lys Asp Glu Leu
```

What is claimed is:

1. A method for producing a lysosomal enzyme which is enzymatically active, comprising:

recovering the lysosomal enzyme from (i) a transgenic plant cell or (ii) a cell, tissue or organ of a transgenic plant, which transgenic plant cell or plant is transformed or transfected with a recombinant expression construct comprising a nucleotide sequence encoding the lysosomal enzyme and a promoter that regulates expression of the nucleotide sequence so that the lysosomal enzyme is expressed by the transgenic plant cell or plant.

2. The method according to claim 1, in which the promoter is an inducible promoter.

3. The method according to claim 2, in which the inducible promoter is induced by mechanical gene activation.

4. The method according to claim 3, in which the inducible promoter comprises SEQ ID NO:5.

5. The method according to claim 2, which is carried out with the transgenic plant and additionally comprises a step of inducing the inducible promoter before or after the transgenic plant is harvested, which inducing step is carried out before recovering the lysosomal enzyme from the cell, tissue or organ of the transgenic plant.

6. The method according to claim 1, in which the lysosomal enzyme is a modified lysosomal enzyme which is enzymatically active and comprises:

(a) an enzymatically-active fragment of a human or animal lysosomal enzyme;

(b) the human or animal lysosomal enzyme or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the human or animal lysosomal enzyme or (a); or

(c) the human or animal lysosomal enzyme or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions.

7. The method according to claim 6, in which the modified lysosomal enzyme comprises a signal peptide or detectable marker peptide at the amino or carboxyl terminal of the modified lysosomal enzyme.

8. The method according to claim 7, in which the modified lysosomal enzyme is recovered from (i) the transgenic plant cell or (ii) the cell, tissue or organ of the transgenic plant by reacting with an antibody that binds the detectable marker peptide.

9. The method according to claim 7, in which the antibody is a monoclonal antibody.

10. The method according to claim 7, in which the detectable marker peptide comprises SEQ ID NO:10.

11. The method according to claim 6, in which the modified lysosomal enzyme comprises:

(a) an enzymatically-active fragment of an α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase;

(b) the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase, sialidase or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase, sialidase or (a); or

(c) the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, -iduronate sulfatase, α-mannosidase, sialidase or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions.

12. The method according to claim 11, in which the modified lysosomal enzyme comprises:

(a) an enzymatically-active fragment of a human glucocerebrosidase or human α-L-iduronidase enzyme;

(b) the human glucocerebrosidase, human α-L-iduronidase or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the human glucocerebrosidase, human α-L-iduronidase or (a); or

(c) the human glucocerebrosidase, human α-L-iduronidase or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions.

13. The method according to claim 6, in which the modified lysosomal enzyme is a fusion protein comprising:

(I) (a) the enzymatically-active fragment of the human or animal lysosomal enzyme,

(b) the human or animal lysosomal enzyme, or

(c) the human or animal lysosomal enzyme or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions, and

(II) a cleavable linker fused to the amino or carboxyl terminus of (I); and the method comprises:

(i) recovering the fusion protein from the transgenic plant cell, or the cell, tissue or organ of the transgenic plant;

(ii) treating the fusion protein with a substance that cleaves the cleavable linker so that (I) is separated from the cleavable linker and any sequence attached thereto; and

(iii) recovering the separated (I).

14. The method according to claim 1, in which the transgenic plant is a transgenic tobacco plant.

15. The method according to claim 1, in which the lysosomal enzyme is a human or animal lysosomal enzyme.

16. The method according to claim 15, in which the lysosomal enzyme is an α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidese.

17. The method according to claim 16, in which the lysosomal enzyme is a human glucocerebrosidase or human α-L-iduronidase.

18. The method according to claim 1, in which the organ is leaf, stem, root, flower, fruit or seed.

19. A recombinant expression construct comprising a nucleotide sequence encoding a lysosomal enzyme and a promoter that regulates the expression of the nucleotide sequence in a plant cell.

20. The recombinant expression construct of claim 19, in which the promoter is an inducible promoter.

21. The recombinant expression construct of claim 20, in which the inducible promoter is induced by mechanical gene activation.

22. The recombinant expression construct of claim 20, in which the inducible promoter comprises SEQ ID NO:5.

23. The recombinant expression construct of claim 19, in which the lysosomal enzyme is a modified lysosomal enzyme which is enzymatically active and comprises:

(a) an enzymatically-active fragment of a human or animal lysosomal enzyme;

(b) the human or animal lysosomal enzyme or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the human or animal lysosomal enzyme or (a); or

(c) the human or animal lysosomal enzyme or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions.

24. The recombinant expression construct of claim 23, in which the modified lysosomal enzyme comprises a signal pertide or detectable marker peptide at the amino or carboxyl terminal of the modified lysosomal enzyme.

25. The recombinant expression construct of claim 24, in which the detectable marker peptide comprises SEQ ID NO:10.

26. The recombinant expression construct of claim 23, in which the modified lysosomal enzyme comprises:

(a) an enzymatically-active fragment of an α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase;

(b) the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase, sialidase or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase, sialidase or (a); or

(c) the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase, sialidase or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions.

27. The recombinant expression construct of claim 26, in which the modified lysosomal enzyme comprises:

(a) an enzymatically-active fragment of a human glucocerebrosidase or human α-L-iduronidase enzyme;

(b) the human glucocerebrosidase or human α-L-iduronidase or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the human glucocerebrosidase, human α-L-iduronidase or (a); or

(c) the human glucocerebrosidase, human α-L-iduronidase or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions.

28. The expression construct of claim 23, in which the modified lysosomal enzyme is a fusion protein comprising

(I) (a) the enzymatically-active fragment of the human or animal lysosomal enzyme,

(b) the human or animal lysosomal enzyme, or

(c) the human or animal lysosomal enzyme or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions, and

(II) a cleavable linker fused to the amino or carboxyl terminus of (I).

29. The recombinant expression construct of claim 19, in which the lysosomal enzyme is a human or animal lysosomal enzyme.

30. The recombinant expression construct of claim 29, in which the lysosomal enzyme is an α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase.

31. The recombinant expression construct of claim 30, in which the lysosomal enzyme is a human glucocerebrosidase or human α-L-iduronidase.

32. A plant transformation vector comprising the recombinant expression construct of claim 19, 20, 24, 29, 31, 23, 27 or 28.

33. A plant which is transformed or transfected with the recombinant expression construct of claim 19, 20, 24, 29, 31, 23, 27 or 28.

34. A plant cell, tissue or organ which is transformed or transfected with the recombinant expression construct of claim 19, 20, 24, 29, 31, 23, 27 or 28.

35. A plant transfection vector comprising the recombinant expression construct of claim 19, 20, 24, 29, 31, 23, 27 or 28.

36. A plasmid comprising the recombinant expression construct of claim 19, 20, 24, 29, 31, 23 or 27.

37. A plasmid CTPro1:hGC:FLAG having the ATCC accession number 97277.

38. A plasmid pCT22 having the ATCC accession number 97701.

39. A plasmid pCT54 having the ATCC accession number 97770.

40. A transgenic plant or plant cell capable of producing a lysosomal enzyme which is enzymatically active, which transgenic plant or plant cell is transformed or transfected with a recombinant expression construct comprising a nucleotide sequence encoding a lysosomal enzyme and a promoter that regulates expression of the nucleotide sequence in the transgenic plant or plant cell.

41. The transgenic plant or plant cell of claim 40, in which the promoter is an inducible promoter.

42. The transgenic plant or plant cell of claim 41, in which the inducible promoter is induced by mechanical gene activation.

43. The transgenic plant or plant cell of claim 42, in which the inducible promoter comprises SEQ ID NO:5.

44. The transgenic plant or plant cell of claim 40, in which the lysosomal enzyme which is a modified lysosomal enzyme which is enzymatically active and which comprises:

(a) an enzymatically-active fragment of a human or animal lysosomal enzyme;

(b) the human or animal lysosomal enzyme or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the human or animal lysosomal enzyme or (a); or

(c) the human or animal lysosomal enzyme or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions.

45. The transgenic plant or plant cell of claim 44, in which the modified lysosomal enzyme comprises a signal peptide or detectable marker peptide at the amino or carboxyl terminal of the modified lysosomal enzyme.

46. The transgenic plant or plant cell of claim 45, in which the detectable marker peptide comprises SEQ ID NO:10.

47. The transgenic plant or plant cell of claim 44, in which the modified lysosomal enzyme comprises:

(a) an enzymatically-active fragment of an α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase;

(b) the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase, sialidase or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase, sialidase or (a); or

(c) the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase, sialidase or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions.

48. The transgenic plant or plant cell of claim 47, in which the modified lysosomal enzyme comprises:

(a) an enzymatically-active fragment of a human glucocerebrosidase or human α-L-iduronidase enzyme;

(b) the human glucocerebrosidase, human α-L-iduronidase or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the human glucocerebrosidase, human α-L-iduronidase or (a); or

(c) the human glucocerebrosidase, human α-L-iduronidase or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions.

49. The transenic plant or plant cell of claim 44, in which the modified lysosomal enzyme is a fusion protein comprising:

(I) (a) the enzymatically-active fragment of the human or animal lysosomal enzyme,

(b) the human or animal lysosomal enzyme, or

(c) the human or animal lysosomal enzyme or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions, and

(II) a cleavable linker fused to the amino or carboxyl terminus of (I).

50. The transgenic plant or plant cell of claim 40, in which the transgenic plant or plant cell is a transgenic tobacco plant or tobacco cell.

51. The transgenic plant or plant cell of claim 40, in which the lysosomal enzyme is a human or animal lysosomal enzyme.

52. The transgenic plant or plant cell of claim 51, in which the lysosomal enzyme is an α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase.

53. The transgenic plant or plant cell of claim 52, in which the lysosomal enzyme is a human glucocerebrosidase or human α-L-iduronidase.

54. A leaf, stem, root, flower or seed of the transgenic plant of claim 40, 41, 45, 50, 51, 53, 44, 48 or 49.

55. A seed of plant line hGC X-11, which seed has the ATCC Accession No. 97275.

56. A seed of plant line hGC X-27, which seed has the ATCC Accession No. 97276.

57. A seed of plant line CT40-9, which seed has the ATCC Accession No. 97700.

58. A plant grown from the seed of claim 55, 56 or 57.

59. A lysosomal enzyme which is enzymatically active and is produced according to a process comprising:

recovering the lysosomal enzyme from (i) a transgenic plant cell or (ii) a cell, tissue or organ of a transgenic plant which transgenic plant cell or plant is transformed or transfected with a recombinant expression construct comprising a nucleotide sequence encoding the lysosomal enzyme and a promoter that regulates expression of the nucleotide sequence so that the lysosomal enzyme is expressed by the transgenic plant cell or plant.

60. The lysosomal enzyme of claim 59, in which the promoter is an inducible promoter.

61. The lysosomal enzyme of claim 60, in which the inducible promoter comprises SEQ ID NO:5.

62. The lysosomal enzyme of claim 60, which process is carried out with the transgenic plant and additionally comprises a step of inducing the inducible promoter before or after the transgenic plant is harvested, which inducing step is carried out before recovering the lysosomal enzyme from the cell, tissue or organ of the transgenic plant.

63. The lysosomal enzyme of claim 59, which is a modified lysosomal enzyme which is enzymatically active and comprises:

(a) an enzymatically-active fragment of a human or animal lysosomal enzyme;

(b) the human or animal lysosomal enzyme or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the human or animal lysosomal enzyme or (a); or

(c) the human or animal lysosomal enzyme or (a) having one or more naturally-occurring amino acid, additions, deletions or substitutions.

64. The lysosomal enzyme of claim 63, in which the modified lysosomal enzyme comprises a signal peptide or detectable marker peptide at the amino or carboxyl terminal of the modified lysosomal enzyme.

65. The modified lysosomal enzyme of claim 64, in which the detectable marker peptide comprises SEQ ID NO:10.

66. The lysosomal enzyme of claim 63, in which the modified lysosomal enzyme comprises:

(a) an enzymatically-active fragment of an α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase;

(b) the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase, sialidase or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase, sialidase or (a); or

(c) the α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfazase, α-mannosidase, sialidase or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions.

67. The lysosomal enzyme of claim 66, in which the modified lysosomal enzyme comprises:

(a) an enzymatically-active fragment of a human glucocerebrosidase or human α-L-iduronidase enzyme;

(b) the human glucocerebrosidase, human α-L-iduronidase or (a) having one or more amino acid residues added to the amino or carboxyl terminus of the human glucocerebrosidase, human α-L-iduronidase or (a); or

(c) the human glucocerebrosidase, human α-L-iduronidase or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions.

68. The lysosomal enzyme of claim 63, in which the modified lysosomal enzyme is a fusion protein comprising:

(I) (a) the enzymatically-active fragment of the human or animal lysosomal enzyme,

(b) the human or animal lysosomal enzyme, or

(c) the human or animal lysosomal enzyme or (a) having one or more naturally-occurring amino acid additions, deletions or substitutions, and

(II) a cleavable linker fused to the amino or carboxyl terminus of (I).

69. The lysosomal enzyme of claim 59, in which the transgenic plant is a transgenic tobacco plant.

70. The lysosomal enzyme of claim 39, in which the lysosomal enzyme is a human or animal lysosomal enzyme.

71. The lysosomal enzyme of claim 70, in which the lysosomal enzyme is an α-N-acetylgalactosaminidase, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase.

72. The lysosomal enzyme of claim 71, in which the lysosomal enzyme is a human glucocerebrosidase or human α-L-iduronidase.

73. The lysosomal enzyme of claim 59, in which the organ is leaf, stem, root, flower, fruit or seed.

* * * * *

# EXHIBIT D

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| Patent Bibliographic Data | | | 06/14/2012 02:17 PM | | |
|---|---|---|---|---|---|
| Patent Number: | 5929304 | | Application Number: | 08713928 | |
| Issue Date: | 07/27/1999 | | Filing Date: | 09/13/1996 | |
| Title: | PRODUCTION OF LYSOSOMAL ENZYMES IN PLANT-BASED EXPRESSION SYSTEMS | | | | |
| Status: | 4th, 8th and 12th year fees paid | | | Entity: | Small |
| Window Opens: | N/A | Surcharge Date: | N/A | Expiration: | N/A |
| Fee Amt Due: | Window not open | Surchg Amt Due: | Window not open | Total Amt Due: | Window not open |
| Fee Code: | | | | | |
| Surcharge Fee Code: | | | | | |
| Most recent events (up to 7): | 01/12/2011<br>01/12/2007<br>11/25/2002 | Payment of Maintenance Fee, 12th Yr, Small Entity.<br>Payment of Maintenance Fee, 8th Yr, Small Entity.<br>Payment of Maintenance Fee, 4th Yr, Small Entity.<br>— End of Maintenance History — | | | |
| Address for fee purposes: | PENNIE & EDMONDS<br>1667 K STREET NW<br>WASHINGTON DC 20006 | | | | |

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EXHIBIT

D

PENGAD 800-631-6989

IN THE U.S. PATENT AND TRADEMARK OFFICE

In re Patent of: VIA FAX: 571-273-8300

Radin et al.

Patent No.: 5,929,304

Issued: Jul. 27, 1999

For: Production of Lysosomal Enzymes in Plant-Based Expression Systems

CHANGE IN ENTITY STATUS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned has noted that the 12th year maintenance fee was paid with a claim of small entity status. The claim of small entity is not correct for this case, and the undersigned wishes to correct the error immediately. The error was discovered when we conducted a review of this patent in preparation of an request for extension of patent term. The owner is a non-profit entity entitled to small entity status as is our licensee of this patent which is a small business with less than 500 employees. We discovered that our licensee, while entitled to small entity status, had sublicensed this patent to a company which does not qualify for small entity status.

Attached is a chart showing (1) the maintenance fee due and fee paid; (2) the amount paid to the USPTO; and (3) the amount owed to the USPTO. I have enclosed a credit card payment form to authorize the charge of $2,675.00 which is the difference between the small entity fee paid and large entity fee due for the 12th year maintenance fee.

Respectfully submitted,

Date: 06/19/2012

Mark S. Coburn

Mark S. Coburn
President
Virginia Tech Intellectual Properties, Inc.

Re: Patent No. 5,929,304

CHART OF FEES OWED

The chart below shows a breakdown of the monies paid to the USPTO and the monies owed to the USPTO.

| Maintenance Fee and Date Filed | Amount Paid to USPTO | Amount Owed to USPTO |
|---|---|---|
| 12th Yr Maintenance Fee<br>Paid: 1/12/2011 | $2,055.00 | $4,730.00 |

Therefore the total fee owed to the USPTO is $2,675.00

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## Credit Card Transactions Report

Date and Time of Report: 06-22-2012 16:11:41 EDT
Start Date: 06-19-2012 End Date: 06-22-2012 Credit Card Number: [redacted]

**DISCLAIMER:** Sale Item Amount column reflects all sale items included in the sale while the Charge Amount column reflects the total amount of the sale that was charged against the Credit Card and any remaining balance was satisfied with another form of payment such as a deposit account or electronic fund transfer.

### Credit Card Sales

| Accounting Date | Create Date | Sale Item Amount | Charge Amount | Fee Code | Name Number | Name Number Cont... | Attorney Docket Number | Stat |
|---|---|---|---|---|---|---|---|---|
| 06/19/2012 | 06/18/2012 | [redacted] | [redacted] | 1601 | [redacted] | | VTIP-64-11137-PCT | Active |
| 06/19/2012 | 06/18/2012 | [redacted] | | 1701 | [redacted] | | VTIP-64-11137-PCT | Active |
| 06/19/2012 | 06/18/2012 | [redacted] | | 1703 | [redacted] | | VTIP-64-11137-PCT | Active |
| 06/19/2012 | 06/18/2012 | [redacted] | | 1709 | [redacted] | | VTIP-64-11137-PCT | Active |
| 06/21/2012 | 06/21/2012 | $2,675.00 | $2,675.00 | 1599 | 5929304 | | | Active |

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### Suspended Payments

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### Replenishment Payments

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# EXHIBIT E

# Cross-Discipline Team Leader Review

| | |
|---|---|
| **Date** | February 24, 2011 |
| **From** | Lynne Yao, M.D. |
| **Subject** | Cross-Discipline Team Leader Review |
| **NDA/BLA #**<br>**Supplement#** | NDA 22-458 |
| **Applicant** | Protalix |
| **Date of Submission** | Dated April 26, 2010; Received April 26, 2010 |
| **PDUFA Goal Date** | February 26, 2011 |
| **Proprietary Name /**<br>**Established (USAN) names** | Elelyso<br>Taliglucerase alfa |
| **Dosage forms / Strength** | Lyophilized powder for solution for injection<br>200 Units/vial [redacted] (b)(4) |
| **Proposed Indication(s)** | Long-term enzyme replacement therapy for patients with type 1 Gaucher disease |
| **Recommended:** | *Complete Response* |

# 1. Introduction

This new drug application (NDA 22-458) was received on April 26, 2010, as an electronic submission. The application is for Elelyso, a formulation of taliglucerase alfa, a new molecular entity. Taliglucerase alfa is a recombinant form of glucocerebrosidase, which is intended "for the long-term enzyme replacement therapy for patients with type 1 Gaucher disease."

Taliglucerase alfa is manufactured using a novel carrot cell expression system; an expression system has not been previously evaluated by the Agency. A stringent review of the complete characterization of the drug substance and product, manufacturing, and controls developed by the applicant to ensure consistency and comparability of final lots of drug product was performed. The CMC review has uncovered several deficiencies in the application relating to the particulate testing and appearance of the drug product, the comparability of product produced after the media was switched (differences in phase 3 trial material compared to the to-be-marketed product), development of an acceptable potency assay for the drug product, process validation for the drug product, and acceptable characterization and release testing of the drug substance and product. Additionally, the product quality microbiology review also noted deficiencies that must be addressed before the application can be approved. These included deficiencies in product bulk hold time, sterilization of lyophilizers used, and microbiological analytical procedures.

The manufacturing facilities for taliglucerase alfa were also inspected, and serious deficiencies were noted by the Office of Compliance in two facilities (Protalix, Ltd., and [redacted] (b)(4)) involved in the manufacturing of taliglucerase alfa. However, the responses to these manufacturing deficiencies submitted by the applicant were reviewed by the Office of Compliance and the current classification for both facilities is voluntary action indicated (VAI). Therefore, the Office of Compliance has recommended that the application is approvable.

Additionally, the application relies upon a single, randomized, dose comparison study. Issues regarding the sufficiency of evidence to support the clinical efficacy compared to other approved enzyme replacement therapies for Gaucher disease will be discussed. Furthermore, the impact of adequate immunogenicity assays in evaluating the potential impact of development of anti-product IgG antibodies on efficacy, safety, and relevant pharmacokinetic and pharmacodynamic parameters will also be discussed.

Therefore, the CMC, product quality microbiology, immunogenicity, pharmacology/toxicology, clinical pharmacology, clinical, and statistical reviewers have all recommended that a complete response action be taken for this application.

This memo documents my concurrence with the review teams' recommendations for a complete response (CR) action.

# 2. Background

**A. Clinical Background**

Gaucher disease is the most common lysosomal storage disease. The incidence of Gaucher disease varies based on ethnicity. The greatest incidence occurs in Ashkenazi Jews, with an estimated incidence of 1:450 to 1:1000. The estimated incidence in non-Jews ranges from 1:20,000 to 1:57,000. Gaucher disease is a chronic multisystem disease resulting from deficient or absent activity of the lysosomal hydrolase glucocerbrosidase. Glucocerbrosidase is an enzyme responsible for the breakdown of glucocerebroside (glucosylceramide). Deficiency or absence of glucocerbrosidase results in the accumulation of glucosylceramide within lysosomes predominately in monocyte-derived macrophages which are most commonly found in spleen, lymph nodes, liver (Kupffer cells), bone marrow, and to a lesser degree in lung, resulting in the clinical consequences of Gaucher disease. Patients with Gaucher disease typically develop hepatosplenomegaly. Hepatic synthetic function is generally preserved, but splenomegaly generally results in anemia and thrombocytopenia due to splenic sequestration. Bone involvement is caused by accumulation of glucosylceramide in bone marrow macrophages and results in decreased osteoblast activity leading osteopenia, and pathologic fractures. Avascular necrosis, bone infarcts, and abnormal bone remodeling can also lead to bone pain crises in Gaucher patients. Pulmonary complications, including interstitial fibrosis are uncommon, but can occur. Gaucher disease is clinically divided into three distinct subtypes, although, there is some overlap in these subtypes; Type 1 disease, or non-neuronopathic disease; Type 2 or acute neuronopathic disease, and Type 3, or sub-acute neuronopathic disease. Neuronopathic disease is caused by accumulation of glucocerebroside within neurons can also lead to accumulation of glucosylsphingosine, a lysosphingolipid derivative. Glucosylsphingosine is toxic to neurons, although this may not be the only mechanism that produces neuronopathic disease.

There are several treatments available for targeting specific therapeutic paths. Enzyme replacement therapies (ERTs) have been developed to replace the deficient enzyme with exogenous glucocerbrosidase. The first ERT approved was Ceredase (alglucerase) (Genzyme, 1991, NDA 20-057). Ceredase is glucocerbrosidase derived from human placenta. Subsequently, Cerezyme (imiglucerase), a synthetic enzyme produced in Chinese Hamster Ovary (CHO) cells (Genzyme, NDA 20-367) was approved in the U.S. in 1994. Ceredase is still available in the U.S., but its use is negligible. Velaglucerase (Shire, NDA 22-575) was approved in February 2010, and is also a synthetic form of glucocerebrosidase that is produced in CHO cells.

Other specific therapeutic strategies include substrate reduction therapy, gene therapy, and bone marrow transplantation. Substrate reduction therapy, or perhaps, more accurately, substrate synthesis inhibition, is an approach that aims to reduce the accumulation of toxic substrates. Zavesca (miglustat) (Actelion Pharmaceuticals, NDA 21-348) was approved in the U.S. in 2003. However, substrate synthesis inhibition therapy with Zavesca is indicated only for patients for whom enzyme replacement therapy is not a therapeutic option (e.g., due to constraints such as allergy, hypersensitivity, or poor venous access). Bone marrow transplantation has been shown to "cure" Gaucher disease but the high mortality and morbidity of the procedure preclude its use and is rarely performed now. Finally, gene therapy is

currently being evaluated, but there are currently no approved gene therapies approved in the U.S. However, if gene therapy proves successful, this treatment strategy may prove to be curative.

**B. Regulatory Background**

**Presubmission Regulatory Activity**

Taliglucerase was developed under IND 69,703. The following list includes highlights of the development of taliglucerase:

- June 30, 2004: Pre-IND meeting held to discuss design of Phase 1 trial (PB-06-001). The meeting minutes state that a single Phase 3 trial to support submission of an NDA could be sufficient if, ". . . the results are sufficiently robust, statistically significant and there is an adequate safety profile."

- June 15, 2005: The initial IND for taliglucerase alfa (IND 69,703) was submitted.

- July 15, 2005: The IND was placed on Partial Hold due to missing nonclinical animal studies (chronic toxicity and reproductive toxicology studies or a single bridging study comparing taliglucerase alfa to Cerezyme).

- Feb 21, 2007: Type B meeting held to discuss partial clinical hold. Agreement was made that 9-month chronic toxicity study in monkeys could serve as the basis to remove partial clinical hold; the study did not have to demonstrate similarity to Cerezyme.

- March 28, 2007: The sponsor (Protalix) submitted a special protocol assessment (SPA) for a proposed phase 3 clinical trial. No agreement was reached on the SPA due to deficiencies in MRI methodology and the statistical analysis plan.

- April 16, 2007: The Partial Hold was removed after review of the 9-month chronic toxicity data. The Division recommended additional nonclinical studies (reproductive toxicology studies) be conducted prior to Phase 3 trials.

- May 21, 2009: Pre-NDA meeting to discuss contents of NDA submission (see Meeting Minutes archived in DARRTS June 29, 2009). Additional comments regarding MRI evaluation of spleen volume, appropriate statistical analyses, and plans for clinical development in the pediatric population were discussed. Additional requirements for the NDA submission relating product quality, nonclinical pharmacology/toxicology, and clinical pharmacology were also discussed.

- July22, 2009: A drug shortage develops because of manufacturing deficiencies at Genzyme's Allston Landing facility that affect the global Cerezyme supply. The applicant submits an expanded access treatment protocol for review.

- August 14, 2009: Expanded access treatment protocol was allowed to proceed.

- August 24, 2009: Fast Track Designation was granted for "the investigation of taliglucerase for treatment of type 1 Gaucher disease."

- Orphan Designation was granted for taliglucerase alfa on September 3, 2009.

- September 14, 2009: Applicant submits NDA as rolling review.

- December 9, 2009: Applicant submits final sections of NDA; however, the application was deemed to be incomplete because of missing CMC data. Therefore, the application is not filed, and the rolling review continued.

- April 26, 2010: Final sections of NDA were submitted by the applicant and review clock was initiated.

**Current Submission and Review**
The application was submitted on April 26, 2010. This submission was granted a standard review.

Clinical Review by C. Epps, dated February 22, 2011
Statistical Review by B. Vali, dated February 24, 2011
Pharmacology/Toxicology Review by T. Chakraborti, dated December 3, 2010
Clinical Pharmacology Review by I.J Lee with concurrence by G. Burkhart, dated January 13, 2011
Product Quality Review (Division of Therapeutic Proteins) by R. Ledwidge, and Product Quality Executive Summary by G. Johnson, with concurrence by B. Cherney and A. Rosenberg, dated February 24, 2011
Product Quality Immunogenicity Review by F. Sheikh, with concurrence by S. Kirshner, dated February 8, 2011
Product Quality Microbiology Review by V. Pawar, with concurrence by B. Riley, dated February 10, 2011
Division of Scientific Investigation Summary by K. Malek, dated January 21, 2011
Division of Medication Error Prevention and Analysis (DMEPA) Proprietary Name Review by Z. Oleszczuk, dated February 1, 2011

# 3. CMC/Device

The reader is referred to the Product Quality Review by R. Ledwidge, dated February 24, 2011 and the Product Quality Executive Summary by G. Johnson, dated February 24, 2011 for complete information.

### A. General product quality considerations

Taliglucerase is produced from genetically modified carrot plant root cells. Taliglucerase alfa has a molecular weight of approximately 60,800 Daltons [redacted]. The amino acid sequence identical to the human glucocerebroside sequence but includes [redacted] amino acids at the N-terminal and C-terminal. These additional amino

acids were introduced by a specific plant expression cassette. [redacted] Therefore, unlike Cerezyme, additional modification of the enzyme to incorporate additional terminal mannose residues is not required. [redacted]

The final drug product is a lyophilized powder and will be diluted in sterile diluent. The excipients of the diluent include mannitol, polysorbate 80, and sodium citrate. The final drug product is stored in glass vials and will be filled in 200 unit [redacted] vials. The applicant states that the drug product is stable for up to 24 months while stored at 2-8°C.

The use of a carrot root cell expression system is novel and this expression system has not previously been evaluated by CMC. The CMC reviewer notes important advantages to this expression system; the growth media is devoid of any mammalian derived products [redacted] which eliminates the risk of mammalian adventitious viral agents, N-glycosylation modification (exposure of terminal mannose residues) can be performed in the plant vacuole and therefore further processing to expose terminal mannose residues are not required with this system.

However, this novel carrot root cell expression system also carries the potential for manufacturing problems that are completely different from traditional mammalian cell-based expression systems. Therefore, stringent review of the complete characterization of the drug substance and product, manufacturing, and controls developed by the applicant to ensure consistency and comparability of final lots of drug product was performed.

Product Quality Review
The CMC reviewer concluded that the information in the application was insufficient at the present time to conclude that the manufacturing of the product is well-controlled and leads to a pure and potent product. Several deficiencies have been identified by the CMC Reviewer that include problems with adequate specifications and assay validation; comparability; process validation; and control of impurities are outlined below:

1. Unacceptable particulate testing and appearance criteria for the drug product
2. Inadequate information to assess development of [redacted] This may have an impact on the comparability of the lots tested in the Phase 3 trial and the to-be-marketed product.
3. Unacceptable potency assay for the drug product
4. Inadequate information to assess the effect of switching to [redacted] on the glycan profile for the drug product.
5. Inadequate process validation for the drug product.
6. Inadequate acceptance criteria for moisture content for the drug product.
7. Inadequate stability testing for the drug product.
8. Inadequate testing for plant specific viruses in the master cell bank.

9. Inadequate description of the process for manufacturing of clinical trial material, and time limits for individual manufacturing steps.
10. Inadequate release testing and characterization of drug substance and drug product.

Based on these deficiencies, the following comments will be provided to the applicant in the complete response letter, and represent deficiencies that must be addressed in their complete response.

<u>Adequate specifications and assay validation</u>

1. Results of USP <788> particulate testing and appearance testing on reconstituted drug product have not been submitted to the NDA. Both tests provide a useful measure of product quality that is not monitored by other tests you have proposed. Add these tests to the release and stability specifications and provide available results for release and stability testing of the three conformance lots and any additional results you may have.

2. A potency assay that quantitatively measures specific receptor binding and/or high affinity internalization into cells is required since internalization is a critical component of taliglucerase alfa's mechanism of action and it is not fully assessed in your current potency assay. The assay should use multiple taliglucerase alfa concentrations to generate a complete dose-response curve in order to calculate the half-maximal effective concentration ($EC_{50app}$). Develop and implement this assay for use in release and stability testing.

3. Some SE-HPLC chromatographs exhibit a [redacted], it should be identified and, if necessary, controlled. Characterize the protein [redacted] and determine whether a control strategy that better monitors this product attribute(s) should be implemented. Provide the results of your analyses and any proposed changes to your specifications.

4. RP-HPLC chromatograms suggest that taliglucerase alfa variants [redacted] The risk to product quality is expected to vary depending on the nature of the variant. Thus, in order to establish an appropriate control strategy, you should identify and control for the quantity of these variants, if present. It may be useful to alter assay conditions or gradients [redacted]. Provide information on the presence of unresolved variants and, if present, provide a revised specification that more accurately quantitates and controls these variants together with supporting data.

5. Enzyme kinetic parameters and specific activity are measured using synthetic p-nitrophenyl-glucopyranoside (pNP-Glc) substrate. pNP-Glc [redacted] Provide enzyme kinetic data to determine the enzyme kinetic parameters, Km and kcat [redacted] on three lots manufactured [redacted]

[redacted] Include a detailed description of the assay, supporting assay qualification data, as well as a justification for why this test should not be added to the release and stability specifications.

6. Stability testing of diluted drug product in infusion bags did not include USP <788> particulate testing or information on the impact of dilution on subvisible particulates that are between [redacted]. USP <788> testing results are critical to mitigate the risk associated with occlusion of small blood vessels and small subvisible particles may pose an immunogenicity risk. Provide USP <788> particulate testing data for in-use stability studies and an analysis of particulates between [redacted].

7. The mannose content specification is based on a MALDI-TOF analysis of taliglucerase alfa. However, the property that is being measured in the MALDI-TOF analysis is mass to charge ratio, not mannose content. Thus, the acceptance criterion should be set around the mass to charge ratio and the mannose content acceptance criterion should be removed from the MALDI-TOF specification. Provide the new specification together with supporting data.

8. The acceptance criterion for moisture content in drug product is [redacted] for both release and stability testing. Release and stability testing results consistently show moisture content to be below [redacted] and no data were submitted indicating that a [redacted] moisture content would not have an adverse impact on product stability throughout the product's dating period. Amend the moisture content acceptance criterion to reflect your manufacturing capability and consideration of any additional knowledge you may have concerning the impact of moisture on product stability and provide the new specification, if appropriate, together with supporting data.

9. Monosaccharide content and glycan structure analysis submitted in the characterization section of the NDA contained inconsistent results. Monosaccharide content analysis on two batches indicated that the [redacted] whereas the glycan analysis data determined that [redacted] of the glycan structures have a [redacted] Provide an explanation for these results or submit data that identify the more accurate analysis using batches made in [redacted]

10. The acceptance criteria for the enzyme kinetic parameters $K_m$ and $V_{max}$ are [redacted] respectively. An analysis of 40 drug substance batches resulted in mean and standard deviations for $K_m$ and $V_{max}$ equal to [redacted] respectively. Consequently, the acceptance criteria appear too wide and should be amended to reflect process capability and clinical experience. Provide the revised specification for enzyme kinetic parameters or your justification as to why your proposal ensures reproducible product potency.

11. In a [redacted] vial drug product fill, the sampling plan calls for [redacted] to be collected for moisture content testing. [redacted] vials are tested and the mean value is reported on the certificate of

analysis. Because the moisture content in an individual vial will vary within any given lot, the proposed sampling plan should provide a reasonable assessment of the variability of the results within a lot. While data from a robust validation study will provide a basis for establishing the sampling plan for the moisture specification, your current sample size and the mean value set as the reportable result are insufficient to assess the moisture content of the final drug product. Submit the revised specification for moisture content with these considerations in mind and provide a justification for your proposal.

12. Chromatograms for drug substance and drug product RP-HPLC analyses contain data from [redacted]. Perform the RP-HPLC analysis such that data from [redacted] from [redacted] is included so that all potential impurities and contaminants can be detected and controlled if necessary. Provide chromatograms where all data are shown [redacted] on lots evaluated in the [redacted] comparability studies.

13. The isoelectric focusing (IEF) assay has acceptance criteria of [redacted] in a pI range of [redacted] reportedly because of assay variation. This level of assay variability is not consistent with the expected validation characteristics for this type of assay. Develop, implement and provide data on a validated IEF method in which the reference standard always produces the same number of bands in a consistent pI range. In addition, each gel should have a quantity of reference standard loaded near the limits of detection to verify the sensitivity of the analysis.

14. The [redacted] assay results are rounded off to the nearest integer which can mask significant differences in [redacted] between lots. Report all [redacted] assay results to two significant digits without rounding off to the nearest integer, revise the acceptance criterion accordingly and submit the revised specification.

15. [redacted]

16. The peptide map specification calls for [redacted] peptide peaks where a countable peak is defined as [redacted] Justify the use of this acceptance criterion in light of the potential amounts of impurities and contaminants that would be acceptable, or revise the criteria for countable peaks. Also, include a revision of the acceptance criteria such that relative peak areas on several selected peptides are specified. Provide the new specification together with supporting data.

17. A host cell protein standard curve is used to determine the levels of host cell proteins in the drug substance. The data from the standard curve is fit to a four parameter logistic regression model even though the data do not reach a plateau and the fitted curve is not fully determined. However, there is a simple linear relationship between host cell protein and assay response. Provide a justification for the use of a four parameter logistic regression model or use a linear regression model to generate a host cell protein standard curve. Submit the revised specification along with the supporting analytical method validation data.

Comparability

18. The relative amounts of the individual glycans in the glycan profile shifted upon the switch to [redacted]. Since the glycan structures are critical to taliglucerase alfa's mechanism of action, a change in the concentration of the glycan structures has the potential to adversely impact clinical performance. Using a potency assay that quantitatively measures specific receptor binding and/or high affinity internalization into cells (see previous comment), perform a head-to-head comparison of three drug substance lots of taliglucerase alfa manufactured in [redacted]

19. Results for SE-HPLC data provided in the NDA are reported as [redacted] As [redacted] may represent a different risk to product quality, they should be independently monitored and controlled. To support your revised acceptance criteria, provide all SE-HPLC data available to date in the application with [redacted] reported separately. For comparison purposes, provide tabulated drug product stability SE-HPLC data separating drug product lots that were manufactured with drug substance made exclusively in [redacted]

20. Your SE-HPLC test method employed a UV detector. However, use of a light scattering detector may allow [redacted] that migrate in the void volume to be observed following SE-HPLC. This provides a much more sensitive qualitative method for monitoring this product attribute. Perform a head-to-head comparison of three drug product lots manufactured exclusively from drug substance made in [redacted] using light scatter detection and provide the results in your resubmission.

Process Validation

21. The time limits for individual manufacturing steps and for the complete manufacturing process are not clearly defined in the NDA. For example, strict limits for [redacted] Provide this information and relate it to the processes used to manufacture clinical study lot PB-06-001, commercial validation lots and the genomic stability sequencing study.

22. Process validation reports indicate that vials containing drug product were put on [redacted] Validation of the lyophilization process should include assessment of vials [redacted] and in different positions within a shelf to confirm consistency of the lyophilization process. Provide a revised validation protocol and report including the results for moisture content testing.

23. [redacted]

Control of Impurities

24. The testing to demonstrate that the master cell bank was free of plant specific viruses tabulated the results without providing data on the suitability of the PCR methods to detect viruses. In order to interpret the results you provided, the suitability of methods for their intended purpose needs to be assessed. Provide the assay qualification data and a description of the system suitability controls for each PCR method used to detect plant specific viruses.

25. The compound [redacted] is a component [redacted] and levels in drug substance or drug product were not determined [redacted] may exhibit toxicity to humans [redacted] and is therefore viewed as a [redacted] impurity that should be well controlled. Provide a control strategy to either include a limit on [redacted] to a level that will not impact product quality as it may relate to safety or efficacy, or validate that the process can clear [redacted] to an appropriate level.

26. [redacted], but its final concentration in drug product has not been determined. The label should accurately describe the final concentration of all excipients which should be confirmed at release. Provide the results on the [redacted] concentration for three drug product lots and provide your

justification for not implementing the determination of [redacted] as a drug product release test.

Conclusions and Recommendations

Based on these deficiencies, the product quality reviewer is recommending a Complete Response Action for this application. I agree with the product quality reviewer's recommendation.

**B. Facilities review/inspection**

The applicant included 10 facilities that are involved in the manufacturing of taliglucerase alfa. Pre-approval inspections were performed at several facilities including Protalix, Ltd. And [redacted], where there has been no previous inspection history. One site, [redacted] has also not had a previous inspection history, but the applicant subsequently removed this site from the application.

There are significant deficiencies noted in the inspections at two facilities; [redacted] and Protalix, Ltd. The [redacted] facility is involved in the manufacture of drug product [redacted] This facility was inspected from [redacted]. Significant deficiencies were noted during the inspection of this facility and were documented in the Establishment Inspection Report (EIR) and in a Form 483 letter that was sent to [redacted]. Furthermore, it was also noted during the inspection that the company failed to open investigations regarding [redacted] of taliglucerase alfa for specific batches used in clinical trials. The presence of [redacted] clearly does not meet the pre-determined acceptance criteria for drug substance. Therefore, Office of Compliance determined that the [redacted] facility's GMP status is Official Action Indicated (OAI) and recommended a Withhold approval action for the application. Furthermore, the Office of Compliance issued an Untitled Letter (UL) to [redacted], stating the following issues that the company must respond to with supporting documentation:

1. Please provide documentation regarding the identification of the [redacted], as well as an impact assessment regarding the presence of [redacted].

2. The in-process test for Appearance (Ic) in the Interim Process Validation Report for batch 004268 states that the [redacted] Please explain why the results page for in-process testing of this process validation batch indicates the batch "complies" with this specification, while the executed batch record for batch 004268 contains notations [redacted]. It should be noted that the batch records for all three process validation batches (004268, 014461, and 016489) contain notations [redacted].

3. The Interim Process Validation Report for batch 004268 also includes a specification for Appearance during the [redacted] The acceptance criteria for appearance is, [redacted] The result for batch 004268 at the [redacted] This does not appear to satisfy your pre-determined acceptance criteria. Please explain why the summary and conclusions section of the Interim Process Validation Report states that, "all in-process and release test results conform with the specifications."

4. Please explain why the Certificates of Conformance for [redacted] 200U (taliglucerase alfa) batches 004268,014461, and 016489 indicate that there were no deviations in the manufacturing and the "agreed part of quality control" for the batches, although [redacted] were noted during the compounding of these batches.
5. The in-process test, [redacted] for batch 016489 failed to meet specifications for [redacted]. The application (NA 22458) specifies the acceptance limits for [redacted] Batch 016489 failed to meet these specifications with a result of [redacted] and what impact this might have on batch 016489.

The applicant provided responses to these deficiencies late in the review cycle. However, the Office of Compliance reviewed the applicant's responses and deemed them to be adequate. Therefore, the official classification of the facility is voluntary action indicated (VAI).

The Protalix facility is involved in the manufacturing [redacted]. This facility was inspected from October 10-17, 2010. The deficiencies noted in the Form 483 letter sent to Protalix, Ltd., include the following observations:

1. Inadequate control of the manufacturing process for taliglucerase alfa. No demonstration that the [redacted]

2. Inadequate investigations and/or corrective actions regarding contaminations during upstream manufacturing of taliglucerase alfa

3. Inadequate risk assessments regarding Bioburden contamination of taliglucerase alfa.

4. Process Validation for taliglucerase alfa is incomplete.

5. Assay validation is incomplete. The level of sensitivity to monitor taliglucerase alfa variants with SEC-HPLC is unknown

6. Components of the taliglucerase alfa manufacturing process are not adequately qualified for their intended use.

7. Inadequate procedures for documenting investigations. The procedures for conducting investigations of out-of-specification test results and deviations do not specify time limits for their completion, and investigations and corrective/preventative actions (CAPAs) are not always timely.

8. Written procedures and batch record are lacking

9. Deficient calibration program for laboratory equipment

10. Deficient calibration program for production equipment

The applicant provided written responses to address all of these deficiencies. The Office of Compliance reviewed the applicant's responses to the 483 letter and determined that the responses were adequate. Therefore, the classification of the facility is voluntary action indicated (VAI).

Conclusions and Recommendations
Based on the VAI status of these two manufacturing facilities and the NAI status of the other facilities involved in the manufacturing of taliglucerase alfa, the Office of Compliance has recommended that the applicant can be approvable. I agree with this compliance recommendation.

**C. Other notable issues:**

**Immunogenicity**
The reader is referred to the Product Quality Immunogenicity Review by F. Sheikh dated February 8, 2011 for complete information.

The assays used to assess immunogenicity of taliglucerase alfa were reviewed. The applicant included validation information for several anti-taliglucerase antibody assays, including IgG, IgE, and neutralizing antibody assay for enzyme activity. The immunogenicity reviewer noted several deficiencies in the anti-taliglucerase antibody assay validation information submitted by the applicant. Additional information to address these deficiencies was sent to the applicant in the 74-day letter. The applicant provided additional information to address some of the deficiencies identified by the immunogenicity reviewer. However, several issues remain outstanding. Specifically, the applicant has not completed the development and validation of an assay to evaluate for neutralizing antibodies for enzyme uptake by macrophages. IgG antibodies for enzyme uptake appear to be the main mechanism for neutralization of activity of ERTs based on data from other approved ERTs. Therefore, development and validation of this assay should be required to fully assess the immunogenicity of taliglucerase alfa.

Additionally, the immunogenicity reviewer disagrees with the criteria used by the applicant to identify patients who develop anti-taliglucerase IgG antibodies. The cut-point chosen by the applicant for the immunodepletion assay used to confirm the presence of anti-taliglucerase IgG

antibodies was determined to be unacceptable. Furthermore, the applicant included patients as having developed IgG antibodies only if the immunodepletion assay was positive at the end of the study. This is not consistent with the definition used in other ERT applications. The Agency has consistently defined patients who develop IgG antibodies as any patient who developed a positive antibody titer *at any time point in the study*, not only the last time point. The immunogenicity reviewer notes that using a more stringent cut-point for the immunodepletion assay may result in an additional 10-13 patients who are determined to have developed anti-taliglucerase alfa antibodies by the end of the phase 3 trial. Therefore, the determination of an appropriate cut-point for the confirmatory immunodepletion assay will be required to clearly establish the efficacy and safety of the product. Finally, the immunogenicity reviewer cited specific issues with the lack of a low level positive control (rabbit anti-taliglucerase alfa IgG) for anti-taliglucerase alfa antibody assay as well as issues with the choice of positive control for the drug tolerance assay (see below). Therefore, the immunogenicity reviewer is unable to fully assess the immunogenicity of the product and the impact of immunogenicity on safety, PK, and efficacy.

The following comments will be provided to the applicant in the complete response letter, and represent deficiencies that must be addressed in their complete response.

1. The concentration of rabbit anti-taliglucerase alfa IgG antibodies [redacted] that you used for the positive control-1 (PC-1) for the anti-product IgG assay quality assessment (binding assay) was high. The agency recognizes that the limit of detection may be different due to affinity differences of the antibodies in the assay. However, in order to ensure reliable performance of the assay, a lower concentration for the positive control that will produce a signal close to the established cut-point of the assay should also be used. Confirm that your assay contains a low concentration positive control that can reproducibly produce a response closer to the established cut-point of your assay.

2. You set the cut-point at [redacted] for the immunodepletion assay to confirm the antibody status of patients. The agency recommends that the confirmatory cut-point be set based on assay precision. Re-establish the immunodepletion assay cut-point based on assay precision using serum from healthy human subjects and from treatment-naïve patients, if available.

3. In your drug tolerance study, you used control antibodies at a concentration o[redacted]l to assess drug tolerance. Your assay is insufficient to address drug tolerance at low concentrations of anti-product antibodies. Repeat your drug tolerance study in the presence of low concentrations of control antibodies.

4. Develop appropriate quality controls in the neutralizing antibody assay and establish acceptance criteria based on these controls.

5. The specificity assessment should be designed to show that the drug product specifically binds to the antibodies induced by the product in human serum in the presence of exogenously added interfering molecules of similar size and charge (e.g., inclusion of IgG in IgE assay development).

6. We recognize that an alternative control for the anti-product IgE antibody assay may be required if a human positive control is not available, and that the detection limit may vary depending on antibody affinity. However, an estimation of assay sensitivity expressed in mass units is necessary to ensure assay suitability and performance for the intended purpose. Determine assay sensitivity and report the results.

Conclusions and Recommendations

Based on these deficiencies, the product quality immunogenicity reviewer is recommending a Complete Response action for this application. I agree with the product quality reviewer's recommendation.

**Product Quality Microbiology**

The reader is referred to the Product Quality Microbiology Consult Review by V. Pawar, dated February 10, 2011 for complete information.

This review evaluated the manufacturing processes relating to product quality microbiology including [redacted]. These steps in the manufacturing process occur at two sites; [redacted]

The reviewer noted several deficiencies in product quality microbiology at these two sites that must be addressed before an approval action can be taken. These deficiencies include the following:

1. [redacted]
3. Inadequate microbiological analytical procedures (i.e., missing validation summary reports for sterility and bacterial endotoxin test methods).

The following comments will be provided to the applicant in the complete response letter, and represent deficiencies that must be addressed in their complete response.

1. With regard to the validation of process [redacted], provide a bioburden data summary to justify this [redacted].
2. For the [redacted] Lyophilizer, provide summary data from three consecutive successful [redacted] with acceptable [redacted] results.
    a. [redacted].

[redacted]

3. Provide validation summary reports for sterility and bacterial endotoxin test methods.

Conclusions and Recommendations
Based on these deficiencies, the product quality microbiology reviewer is recommending that the application cannot be approved until these issues have been satisfactorily addressed. I agree with the product quality reviewer's recommendation.

# 1. Nonclinical Pharmacology/Toxicology

The reader is referred to the Pharmacology/Toxicology Review by T. Chakraborti, dated December 3, 2010 for complete information.

The application included the following Good Laboratory Practices (GLP) safety pharmacology/toxicology studies that were reviewed:
1. Acute single dose toxicity studies in mice and cynomolgus monkeys
2. One 4-week, repeat dose study in cynomolgus monkeys
3. One 39-week, repeat dose study in cynomolgus monkeys
4. One Segment I fertility and early embryonic development study in Sprague Dawley (SD) rats
5. One Segment II teratology study in rats and New Zealand white rabbits

Additionally, two non-GLP studies were also performed and reviewed by the nonclinical reviewer. These studies included one 14-day repeat dose study in marmoset monkeys, one 29-day repeat dose study in marmoset monkeys.

The nonclinical program evaluated doses of taliglucerase about 14 times the maximum proposed clinical dose of 60 U/kg (60 U/kg is equivalent to 2 mg/kg). In acute toxicity studies in mice and monkeys, taliglucerase alfa was non-lethal at the maximum doses tested (18 mg/kg, or nine time the maximum proposed clinical dose). For the chronic repeat dose and reproductive toxicology studies the dose and drug product used were comparable to the drug product used in the phase 3 clinical trial. A no observed adverse effect level (NOAEL) was noted to be 11mg/kg/day (approximately six times the maximum clinical dose) in a non-GLP, 29-day, repeat dose study in marmoset monkeys. Anti-taliglucerase antibodies were detected in some animals, but neutralizing antibodies were not detected based on the assay used. A NOAEL was noted to be 27.8 mg/kg (about 14 times the maximum clinical dose) in a GLP, 28-day, repeat dose study in cynomolgus monkeys. A target organ of toxicity in this study could not be identified. Similar to the marmoset monkey study, anti-taliglucerase antibodies were detected in some animals, however, none of the animals developed neutralizing antibodies based on the assay used.

Segment I fertility and embryonic development study was performed in SD rats. No treatment related adverse effects were noted in either sex. A Segment II teratology study was performed in both rats and rabbits and no findings were noted. Thus, the nonclinical reviewer concluded that taliglucerase is not teratogenic.

Genetic toxicology and carcinogenicity studies were not conducted or required because this product is a biologic product.

The nonclinical reviewer also provided recommendations for specific sections of product labeling (i.e., section 8.1 Pregnancy, section 8.3 Nursing Mothers, and section 13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility). These sections were not negotiated with the applicant during this review cycle.

Conclusions and Recommendations
The nonclinical reviewer concluded that the nonclinical program for taliglucerase was adequate that that there were no significant safety concerns uncovered in the nonclinical program. Thus, based on the results of the nonclinical studies, there appears to be no significant safety concerns at the intended clinical doses for the proposed indication. Additionally, there are no outstanding nonclinical studies that need to be addressed. I agree with the nonclinical reviewer's conclusions.

# 6. Clinical Pharmacology/Biopharmaceutics

The reader is referred to the Pharmacology/Toxicology Review by I.J. Lee, dated January 13, 2011 for complete information.

The applicant included a phase 1 pharmacokinetic (PK) study report P-01-2005 in healthy subjects and a phase 3 PK study report PB-06-001 in type 1 Gaucher patients for review in this submission.

General clinical pharmacology/Biopharmaceutics
The PK parameter values of taliglucerase alfa were determined in Gaucher patients at the proposed indicated dose of 60 U/kg every other week, and 30 U/kg every other week using a non-compartmental method (see Table 1).

**Table 1: Pharmacokinetic parameters (mean ± standard deviation) of taliglucerase alfa determined in Gaucher patients (Study PB-06-001)**

| Dose Group (Units/kg) | 30 | | 60 | |
|---|---|---|---|---|
| Study Visit | Day 1 | Week 38 | Day 1 | Week 38 |
| Number of Patients | 10 | 14 | 16 | 15 |
| $AUC_{last}$ (ng·hr/mL) | 2,229 ± 669 | 2,654 ± 2,130 | 6,349 ± 2,200 | 7,665 ± 4,578 |
| $AUC_{\infty}$ (ng·hr/mL) | 2,244 ± 674 | 2,706 ± 2,270 | 6,383 ± 2,229 | 8,095 ± 5,087 |
| Extrapolation (%) | 0.64 ± 0.40 | 0.90 ± 1.43 | 0.46 ± 0.39 | 3.25 ± 5.28 |
| $AUC_{last}$/Dose (ng·hr/mL)/mg | 39.1 ± 13.2 | 42.2 ± 30.4 | 54.3 ± 18.9 | 63.4 ± 33.9 |
| CL (L/hr) | 29.4 ± 13.9 | 30.7 ± 14.5 | 20.5 ± 7.1 | 19.9 ± 9.6 |
| Vz (L) | 17.5 ± 11.1 | 16.8 ± 12.7 | 11.7 ± 4.5 | 14.4 ± 6.6 |
| t½ (min) | 25.9 ± 11.8 | 25.1 ± 15.5 | 25.0 ± 10.1 | 36.3 ± 22.8 |

(copied from clinical pharmacology review, I.J. Lee, page 3)

The clinical pharmacology reviewer noted that the PK parameters for taliglucerase alfa do not appear to be dose-proportional based on the doses studied. The AUC values for the 60 U/kg dose compared to the 30 U/kg dose increased by 178% and 200% on Day 1 and Week 38. Additionally, CL values were reduced by 30% and 35% on Day 1 and Week 38, respectively. The clinical pharmacology reviewer also noted that the design of this PK study did not collect samples at or around 90 minutes (the length of the infusion), and therefore, Cmax could not be measured based on this study.

The applicant also performed a phase 1 study in healthy subjects. The utility of the information obtain in this study is unclear because the PK and PD findings in healthy human subjects who presumably have normal glucocerbrosidase activity would like be different than Gaucher patients. The clinical pharmacology reviewer noted that the $AUC_{last}$ and clearance of taliglucerase alfa were both higher (2.6 and 2.8-fold higher, respectively) in healthy subjects compared to Gaucher patients. Additionally, t½ values were longer in Gaucher patients compared to healthy subjects.

The clinical pharmacology reviewer also noted that there appears to be no significant exposure-response relationship based on the two doses of taliglucerase studied (30 U/kg and 60 U/kg) in the primary efficacy endpoint, spleen volume. There was a statistically significant decrease in spleen volume at the end of the study for both doses studied (see section 8: Clinical/Statistical: Efficacy).

<u>Drug-drug interactions</u>
No drug-drug interaction studies were conducted for this submission.

Pathway of elimination
Taliglucerase alfa, a therapeutic protein product, is likely cleared by absorption into macrophages. Therefore, metabolism, excretion, and mass balance studies were not required or performed.

Demographic interactions/intrinsic factors, special populations
The applicant did not explore the impact of intrinsic factors on taliglucerase alfa exposure-efficacy or safety response relationships in the submission. Specifically, the submission did not include studies evaluating geriatric or pediatric or pediatric age groups. The effect of gender, race, and presence of renal or hepatic impairment on PK was also not specifically evaluated in any study.

QT assessment
A thorough QT assessment was not performed nor required because as a therapeutic protein product, taliglucerase alfa, like other ERTs, would not be expected to affect hERG channels. Blockade of hERG channels can result in significant alterations in ventricular repolarization, potentially resulting in torsades de pointe which can degenerate into fatal ventricular arrhythmias. Additionally, ECG results at all visits were normal in a PK study (P-01-2005) in healthy subjects.

Other issues: Immunogenicity
The effect of immunogenicity on PK and PD parameters could not be reviewed during this review cycle because the formation of anti-taliglucerase alfa antibodies could not be adequately determined based on the review of the CMC immunogenicity reviewer (see section 3.C: CMC, above). Additionally, the effect of neutralizing antibodies to enzyme uptake could not be evaluated because the applicant has not yet developed an assay for this specific inhibitory antibody. Information requests regarding the impact of immunogenicity on PK and efficacy of taliglucerase were conveyed to the applicant on November 23 and December 21, 2010. However, there was insufficient time in during the current review cycle to adequately review this information. Furthermore, a clear determination of the anti-taliglucerase antibody assay cut-points has not been established. Therefore, the effect of immunogenicity of PK parameters and efficacy cannot be determined until these assay cut-points have been established.

The following comments will be provided to the applicant in the complete response letter, and represent deficiencies that must be addressed in their complete response.

1. The immunogenic potential of taliglucerase alfa and its impact on the pharmacokinetic and pharmacodynamic (PK and PD) parameters cannot be adequately evaluated.
    a. Propose an acceptable confirmatory cut-point for your anti-product IgG antibody assay and submit a re-analysis of the impact on PK and PD parameters in patients treated with taliglucerase alfa.
    b. Develop an acceptable neutralizing antibody assay and submit a re-analysis of the impact on PK and PD parameters in patients treated with taliglucerase alfa.

Conclusions and Recommendations
The clinical pharmacology reviewer concluded that a complete review of the information was not possible during the current review cycle. Major outstanding issues include the development of a validated assay to assess for neutralizing antibodies for enzyme uptake, and the establishment of anti-taliglucerase antibody confirmatory assay cut-points that can be used to establish the effect of immunogenicity on PK parameters and efficacy. I agree with the clinical pharmacology reviewer's conclusions and recommendations. These issues must be addressed by the applicant in their Complete Response.

# 7. Clinical Microbiology

Clinical microbiology considerations do not apply to this application because taliglucerase alfa is not intended as an antimicrobial product.

# 8. Clinical/Statistical- Efficacy

The reader is referred to the clinical review by C. Epps, dated February 22, 2011, and the statistical review by B. Vali, dated February 24, 2011, for complete information.

The data submitted to support the efficacy of Taliglucerase alfa was contained in a single phase 3 trial, PB-06-001. Additional preliminary supportive efficacy data from two on-going studies were also submitted. Study PB-06-002 is a multicenter, open-label, switch over study in stable Gaucher patients receiving imiglucerase (Cerezyme). The applicant submitted preliminary data on six patients currently enrolled in this study. Study PB-06-003 is an open-label extension study for patients previously enrolled in either study PB-06-001 or PB-06-002. However, the efficacy data from these two studies is insufficient to provide any real conclusions because of the limited number of patients with data provided by the applicant at the time of the submission. Additionally, these studies are on-going and only interim data could be provided. Therefore, the discussion of efficacy will be limited in this review to results from study PB-06-001.

**Study PB-06-001**
Study PB-06-001 was a multicenter, randomized, double-blind, parallel-dose study of 33 adult patients with type 1 Gaucher disease.

Eligibility, treatment and assessments
Enrollment was restricted to patients 18 years of age and older who were naïve to enzyme replacement therapy had a spleen volume as measured my MRI of at least 8 times normal, and thrombocytopenia. Patients were randomized 1:1 to receive taliglucerase at a dose of either 30 units/kg or 60 units/kg every other week for nine months.

Endpoints
The pre-specified primary endpoint was the percent change from baseline in spleen volume as measured by MRI after nine months. The primary efficacy analysis was based on two one-sample t-tests (one for each treatment group) using an alpha level of 0.025.

Major secondary endpoints included the change from base line in hemoglobin, liver volume as measured by MRI as a percent change, and platelet count. Additionally, change from baseline in Gaucher disease biomarkers, chitotriosidase, and PARC/CCL18 were also evaluated. These secondary endpoints were analyzed in a sequential (step-down) approach based on the statistical significance of the primary endpoint and each secondary endpoint in the order presented (i.e., hemoglobin, liver volume, and platelet count) using a one-sample t-test. For both the primary and major secondary endpoint analyses, a mixed effects model was fit to examine whether any differences were noted between dose groups at month 6 and month 9. Patients who withdrew early from the study were analyzed using the last observation carried forward (LOCF) approach. However, if the patient withdrew due to a serious adverse event, no-change from baseline was imputed.

Results
The applicant reports that 33 patients were enrolled and randomized into the study. All but one patient received at least one dose of study drug, and 29/33 patients completed all 20 study visits. Three patients discontinued the study; two withdrew due to adverse events (one each in the 30 unit/kg and 60 unit/kg groups), and one withdrew due to pregnancy (60 unit/kg group). There were 24 patients with reported protocol deviations. Of these, 16 patients had 31 protocol deviations that were approved by the Medical Director, including visits outside the scheduled window or other study procedures that were not performed according to the protocol schedule. Eight patients had protocol deviations that were not approved by the Medical Director including antibody testing that was not done, improper dilution of study drug, positive urine pregnancy test (see above). Additionally, there were 4 patients included in the study that did not meet inclusion criteria but were included by the Medical Director (2 patients with glucuocerebrosidase activity levels that were > 3 nmol/mg*hr, and 2 patients with platelet counts > 120,000 $mm^3$. None of these deviations appear to have affected the outcome of the study results.

The applicant provided demographic data analyses for 31 patients who comprised their intent to treat (ITT) population, defined as patients who received at least one dose of medication and had at least the screening/baseline MRI evaluation. Two patients were excluded from the applicant's ITT population; one patient who withdrew for personal reasons prior to receiving any study medication, and one patient who developed anaphylaxis during the first dose of taliglucerase alfa. It should be noted, however, that the statistical reviewer analyzed the efficacy data using a "corrected intent-to-treat" population (all patients who were randomized into the study), or a corrected intent-to-treat population of 33 patients (16 patients in the 30U/kg arm and 17 patients in the 60U/kg arm). There were no significant differences in mean or median results between treatment groups for age, gender, race, or weight. The majority of patients enrolled were Caucasian (100% for the 30 unit/kg group and 94% for the 60 unit/kg group).

The primary efficacy endpoint was evaluated for both treatment groups at 6 months and at 9 months. Treatment with either 30 U/kg or 60 U/kg was associated with a statistically significant decrease in the percent change in mean spleen volume at 6 months and at 9 months (see Table 2). Both treatment arms demonstrated a decline in spleen volume of greater than 20% (the pre-specified definition of a clinically meaningful change in spleen volume) at both 6 months and 9 months. Furthermore, when these dose arms are compared utilizing a two independent sample t-test in the "corrected in-to-treat" population as defined by the statistical reviewer, a significant difference between the arms is also recognized. The p-value from this two independent sample t-test, which assumes equal variance between the two arms, equals 0.01. The mean difference between these dosing arms (60 units/kg – 30 units/kg) in percent change from baseline in spleen volume is -10.6 with corresponding 95% C.I.(-18.82, -2.27). Although the result from this dose comparison is exploratory in nature, this analysis suggests greater effectiveness of the 60 unit/kg dose (see Figure 1). There were no substantive differences in the analysis when these two patients are included in the analysis.

**Table 2: Mean change in spleen volume by treatment group based on applicant's ITT population**

| Treatment Group | Length of Treatment | |
|---|---|---|
| | 6 months | 9 months |
| **30U/kg Group (n=15)**<br>Mean Percent Change±SD<br>(Min-Max) | -22%±5<br>(-12 to -29)<br>p<0.0001*<br>95% CI (-24.3, -19.7) | -27%±8<br>(-16 to -43)<br>p<0.0001*<br>95% CI (-30.9, -23.1) |
| **60 U/kg Group (n=16)**<br>Mean Percent Change±SD<br>(Min-Max) | -30%±13<br>(+3 to -53)<br>p<0.0001*<br>95% CI (-36.2, -23.8) | -38%±9<br>(-21 to -56)<br>p<0.0001*<br>95% CI (-42.6, -33.4) |
| **All Treatment Groups (n=31)**<br>Mean Percent Change±SD | -26%±10 | -33%±10 |

* p-value obtained from one-sample t-test (combined)
(table modified from clinical review by C. Epps)

**Figure 1: Change in spleen volume by treatment group***

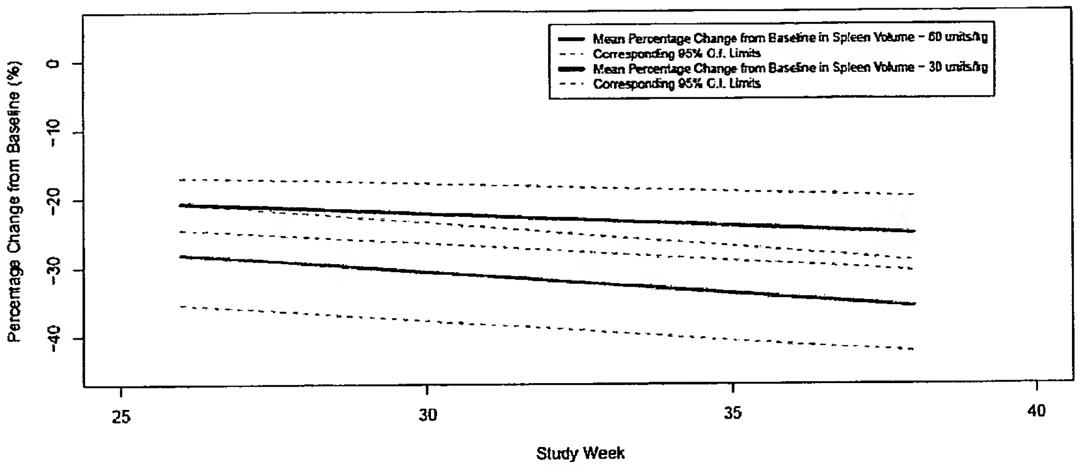


*Figure based on data from "corrected ITT" population
(copied from statistical review by B. Vali)

The clinical and statistical reviewer both concluded that the data demonstrated a clinically meaningful and statistically significant difference in spleen volume in both treatment groups at the end of the study.

Key secondary endpoints for the study include change in liver volume, platelet count, hemoglobin and chitotriosidase levels (see Table 3). There was a statistically significant decrease in the percent change in liver volume compared to baseline for both treatment groups at the end of the study (-11% in the 30 U/kg group and -11% in the 60 U/kg group). Additionally, improvements in mean platelet count and hemoglobin were also demonstrated in both treatment groups at the end of the 9-month treatment period. Finally, decreases in a relevant Gaucher biomarker, chitotriosidase, were also noted in both treatment groups at the end of the study. It should be noted that the improvement in platelet count was only modest in the 30 U/kg group at the end of the study. This difference bordered on statistical significance (p=0.046), however the clinical significance of this differences is not clear. The difference in platelet count in the 60 U/kg group appears to be both clinically meaningful and statistically significant. The clinical relevance of an improvement of 1.6-2.2 g/dL in mean hemoglobin could also be questioned; however, baseline hemoglobin levels for patients in both treatment groups was near normal (12.2 g/dL for the 30 U/kg group and 11.4 g/dL in the 60 U/kg group) and therefore, improvements would be expected to be modest. Overall, these results support the effect of taliglucerase in patients with type 1 Gaucher disease and are consistent with the findings demonstrated in the primary endpoint.

**Table 3: Results of key secondary endpoints at 9 months**

| Secondary Endpoints | 30 U/kg Group | 60 U/kg Group | All Treatment Groups |
|---|---|---|---|
| **Mean percent change in Liver volume** | **-11±11 (p=0.0041)** | **-11±7 (p<0.0001)** | **-11±9** |
| **Mean change in platelet count** | **11K±20K (p=0.046)** | **41K±47K (p=0.0031)** | **27K±39K** |
| **Mean change in hemoglobin** (g/dL) ± SD | **1.6±1.4 (p=0.001)** | **2.2±1.4 (p<0.0001)** | **1.9±1.4** |
| **Mean change in chitotriosidase level** | **-13264±8378 (p<0.0001)** | **-12165±12064 (p=0.016)** | |

p-values obtained from one-sample t-test
(table modified from clinical review by C. Epps)

Effect of Immunogenicity on Efficacy

An important consideration with all enzyme replacement therapies for lysosomal storage diseases is the development of immune responses to the infused enzyme replacement therapy. These immune responses can be associated with the development of allergic/hypersensitivity reactions as well as altered effectiveness of treatment. All patients were tested for development of anti-taliglucerase antibody formation during the study. The applicant noted that only two patients developed anti-taliglucerase IgG antibodies during the study. Both of the patients completed the study and there was no apparent association between the development of anti-taliglucerase IgG antibodies and changes in efficacy outcome. It should also be noted that the immunogenicity reviewer noted five patients (not two, as the applicant stated) with positive anti-taliglucerase IgG antibodies (see immunogenicity reviewer's review for details). However, due to the small numbers of patients reported and the relatively lack of long-term efficacy data, it is not possible to draw clear conclusions regarding the effect of IgG antibody development and long-term efficacy.

Additionally, the immunogenicity reviewer disagrees with the criteria used by the applicant to identify patients who develop anti-taliglucerase IgG antibodies. The cut-point used by the applicant in the confirmatory immunodepletion assay was determined to be unacceptable. Furthermore, the applicant included patients as having developed IgG antibodies only if the assay was positive at the end of the study. This is inconsistent with the definition used in other ERT applications. The Agency has consistently defined patients who develop IgG antibodies as any patient who developed a positive antibody titer *at any time point in the study*, not only the last time point. It is likely that a change in the criteria used to define patients who develop anti-taliglucerase IgG antibodies will increase the number of patients that have developed IgG antibodies and re-analysis of the efficacy and safety data will be warranted. The immunogenicity reviewer notes that in a preliminary analysis of the immunodepletion assay results submitted by the applicant, if a cut-point for the assay consistent with other ERT approvals is used, the number of patients identified as anti-taliglucerase antibody positive increases to 10-13 patients. This increase could have an important effect on the review of the

safety and efficacy data for the product. Furthermore, the applicant has not completed the development and validation of an assay to evaluate for neutralizing antibodies for enzyme uptake by macrophages. IgG antibodies for enzyme uptake appear to be the main mechanism for neutralization of activity of ERTs based on data from other approved ERTs. Therefore, development and validation of this assay should be required to fully assess the immunogenicity of taliglucerase alfa.

Therefore, due to inability to adequately assess the immunogenicity of taliglucerase because of the lack of an acceptable IgG anti-taliglucerase IgG antibody cut-point for the confirmatory immunodepletion assay, lack of an acceptable definition for patients who develop anti-taliglucerase IgG antibodies, and the lack of a validated assay to identify neutralizing antibodies for enzyme uptake, the efficacy and safety of the product cannot be adequately established and I agree with the immunogenicity reviewer recommendation that a complete response action should be taken. Recommendations for the applicant in the complete response letter will include a requirement to reassess the effect of immunogenicity on efficacy and safety after an acceptable criteria for use of the anti-taliglucerase IgG antibody assay are determined.

<u>Conclusions and Recommendations</u>
The data from this single pivotal trial demonstrate that treatment with taliglucerase alfa produced a statistically significant and clinically meaningful decrease in spleen volume at 6 and 9 months. Additionally, important secondary endpoints including decrease in liver volume, and improvements in hemoglobin and platelet count were also demonstrated. Indeed, as was noted in the primary clinical review, the magnitude of the treatment effect for decrease in spleen volume was similar to that of a recently approved ERT for Gaucher disease, velaglucerase. Thus, despite the reliance on a single pivotal trial, the data demonstrate a clear and robust treatment effect that would not have been expected to occur without treatment. In the Guidance for Industry, "Providing clinical Evidence of Effectiveness for Human Drug and Biological Products," specific recommendations regarding the quality and quantity of evidence required to substantiate effectiveness of a product based on a single pivotal trial are discussed. These factors include consistency across study subsets, multiple endpoints involving different events that all demonstrate a statistically persuasive effects, and statistically very persuasive findings in the primary endpoint that would be highly inconsistent with the null hypothesis. All of these factors have been demonstrated in support of the efficacy of taliglucerase alfa based on the single pivotal trial, PB-06-001.

However, the guidance also discusses the important caveats to the reliance of a single pivotal study, including that even a strong result can represent an isolated or biased result. Additionally, the applicant did not perform a head-to-head comparison between taliglucerase and other approved ERTs for Gaucher disease. Therefore, it is not possible to draw clear conclusions about the relative effectiveness of taliglucerase alfa in the treatment of Gaucher disease compared to other approved ERTs. Because Gaucher disease is a life-threatening condition for which there are already approved products in the U.S., lack of clear data demonstrating the relative effectiveness of taliglucerase compared to these approved products is a significant weakness in this application. Furthermore, the clinical reviewer noted two recent publications that suggest that clinical parameters of Gaucher disease remained stable or

did not deteriorate precipitously up to 6 months after treatment withdrawal.[1,2] In the earlier survey, some patients were clinically stable after more than two years without ERT treatment. These data suggest that a trial to evaluate the relative efficacy of taliglucerase compared with other approved ERTs must include data for at least 6-12 months. Additionally, in the approval for VPRIV, the reviewer noted that a head-to-head trial was conducted comparing efficacy and safety of VPRIV compared to Cerezyme in a trial lasting 12 months.

Additionally, the effect of immunogenicity on efficacy could not be fully evaluated during this review cycle because the criteria used by the sponsor to define patients with anti-taliglucerase IgG antibodies was unacceptable. Furthermore, development of neutralizing antibodies to enzyme uptake is a major mechanism by which efficacy can be affected in ERTs. The applicant has not yet developed a validated assay to measure the development of neutralizing antibodies for enzyme uptake for taliglucerase.

I agree with the primary clinical reviewer and statistical reviewer that demonstration of efficacy compared to other approved ERTs has not been established and precludes approval of this product during this review cycle. However, I do not agree with the clinical reviewer and statistical reviewer's recommendation that an additional clinical study must be performed using a head-to-head design to establish the relative efficacy and safety of taliglucerase alfa to other approved ERTs as a condition for resubmission. I recommend that data from study PB-06-002 and PB-06-003 be carefully evaluated to assess the clinical outcome of patients switched from Cerezyme to taliglucerase alfa who have received treatment for at least 12 months. These data may be sufficient to demonstrate the relative effectiveness of taliglucerase alfa compared to Cerezyme. However, if these data are not sufficient to conclude that the efficacy and safety of taliglucerase alfa is similar to Cerezyme, then additional clinical studies may be required in the future. Additionally, a re-analysis of information regarding the immunogenicity of the product and its effect on efficacy and safety must also be included in a re-submission.

# 9. Safety

The safety data for taliglucerase alfa comes from the experience in Study PB-06-001, as well as interim data from Study PB-06-002 (imiglucerase switch study), PB-06-003 (phase 3 extension study), and PB-06-004 (expanded access protocol). The interim data from Studies PB-06-002 and PB-06-003 were minimal, and therefore, the safety data from each study were reviewed separately by the clinical reviewer. Additionally, this product is a new molecular entity and has not been approved outside the U.S. Therefore, there are no postmarketing data available for the safety review.

The safety database consisted of 83 patients with type 1 Gaucher disease. Of the 83 patients, 39% (32/83) were treatment naïve patients who received taliglucerase alfa, and 30% were

1 Elstein D, Abrahamov A et al, Withdrawal of enzyme replacement therapy in Gaucher's disease, *Br. J Haematol* 2000; 110(2):488-492.

2 Zimran A, Altarescu G, Elstein D, Nonprecipitous changes upon withdrawal from imiglucerase for gaucher disease because of a shortage in supply, *Blood Cells Mol Dis* 2011: 46 (1):111-114.

switched from imiglucerase to taliglucerase. Additionally, 44% (27/83) patients completed 12 months of treatment; and only 8% (7/83) completed 24 months of treatment (see Table 4).

Table 4: Total patient exposure to taliglucerase alfa

| | | | Months of Treatment | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Study | 0* | | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 | 27 | 30 | 33 | 36 |
| PB-06-001 | 32 | | 31 | 29 | 29 | | | | | | | | | |
| PB-06-002 | 25 | | 24 | 20 | 8 | | | | | | | | | |
| PB-06-003 | | | | | | 27 | 26 | 20 | 11 | 7 | 3 | 2 | 1 | |
| PB-06-004 | 26 | | 16 | 6 | | | | | | | | | | |
| Total | 83 | | 71 | 55 | 37 | 27 | 26 | 20 | 11 | 7 | 3 | 2 | 1 | |

*Number of subjects enrolled and treated as of June 30, 2010

(copied from Clinical Review by C. Epps)

No deaths were reported. The applicant reported three serious adverse events. These adverse events included one episode each of epistaxis, immune thrombocytopenic purpura (ITP), and nephrolithiasis. The clinical reviewer agreed with the applicant's assessment that these serious adverse events were not likely related to treatment with taliglucerase alfa. However, it is not clear whether the patient with ITP could have developed an immune response during treatment with taliglucerase alfa that could have precipitated this adverse event.

Anaphylaxis, acute and chronic immune reactions, and infusion reactions are the most serious adverse events related to treatment with ERTs for lysosomal storage diseases. The applicant reported only one patient has having developed anaphylaxis. However, the clinical reviewer independently assessed the adverse events and recoded these events based on the clinical definition of anaphylaxis as published by Sampson et al. Based on the clinical reviewer's assessment, 9% (3/32) patients definitely met criteria for anaphylaxis. One patient who developed a "hypersensitivity reaction" may have also had anaphylaxis, but there was insufficient information provided to clearly determine whether this patient sustained a clinical episode of anaphylaxis. In the clinical review for VPRIV, there was one case of anaphylaxis identified in the phase 3 trials. The incidence of anaphylaxis in Cerezyme is estimated to be less than 1% based on a review of the post-marketing experience. It appears that the incidence of anaphylaxis may be slightly higher for taliglucerase alfa, but no clear conclusions can be drawn regarding the relative incidence of anaphylaxis between these products because the data are limited.

Infusion reactions (adverse reactions that occurred during or within 2 hours of the completion of the infusion) were seen commonly with taliglucerase treatment. The clinical reviewer noted that 41-48% of patients across studies PB-06-001, PB-06-002, and PB-06-003 developed infusion reactions. These reactions included abdominal/epigastric discomfort, arthralgia, asthenia chest pain/discomfort, dizziness, drug eruption, dyspnea, erythema, fatigue, feeling

hot, flushing, infusion related reaction, headache, hyperglycemia, hyperhidrosis, hypersensitivity, hypertension, lethargy, nausea, pain, palpitations, presyncope, pruritis, pyrexia, rash/skin irritation, swelling, tachycardia, and vomiting (see Table 6).

**Table 5: Commonly reported infusion reactions across trials for taliglucerase alfa**

| System Organ Class<br>Preferred Term | PB-06-001<br>N=32 | PB-06-002<br>N=25 | PB-06-003<br>N=29 |
|---|---|---|---|
| **General Disorders and Administration Site Conditions (N=13)** | | | |
| Infusion related reaction | 0 | 4 (16%) | 2 (7%) |
| Asthenia | 0 | 2 (8%) | 0 |
| Chest pain/discomfort | 2 (6%) | 0 | 0 |
| Fatigue | 0 | 1 (4%) | 2 (7%) |
| **Nervous System Disorders (N=13)** | | | |
| Headache | 5 (16%) | 2 (8%) | 1 (3%) |
| Hypoesthesia/paresthesia | 1 (3%) | 1 (4%) | 1 (3%) |
| Dizziness/Presyncope | 1 (3%) | 0 | 1 (3%) |
| **Skin and Subcutaneous Tissue Disorders (N=10)** | | | |
| Erythema | 1 (3%) | 1 (4%) | 2 (7%) |
| Urticaria/rash/drug eruption | 0 | 1 (4%) | 3 (10%) |
| Pruritis | 1 (3%) | 1 (4%) | 0 |
| **Gastrointestinal Disorders (N=8)** | | | |
| Abdominal/Epigastric discomfort | 1 (3%) | 1 (4%) | 1 (3%) |
| Diarrhea | 1 (3%) | 1 (4%) | 1 (3%) |
| Vomiting | 1 (3%) | 0 | 1 (3%) |
| **Musculoskeletal and Connective Tissue Disorders (N=7)** | | | |
| Arthralgia/back pain | 2 | 2 (8%) | 3 (10%) |
| **Vascular Disorders (N=6)** | | | |
| Flushing | 2 (6%) | 1 (4%) | 0 |
| Hypertension/increased blood pressure | 1 (3%) | 0 | 2 (7%) |
| **Immune System Disorders (N=3)** | | | |
| Hypersensitivity* | 2 (6%) | 0 | 1 (3%) |

(copied from clinical review, C. Epps)

Common adverse events, defined as occurring in >5% of treated patients, were classified by the clinical reviewer for Study PB-06-001. The most common adverse events included upper respiratory infections/colds (22%) pharyngitis /throat infection, headaches (each 19%), and influenza/flu and arthalgia/back pain (13%) (see Table 6). It should be noted that this study was not placebo-controlled and therefore clear associations between treatment and adverse events are difficult to assess. However, I agree with the clinical reviewer's assessment that adverse reactions could reasonably be defined as those adverse events occurring at an incidence of >5% of treated patients. Common adverse events for studies PB-06-002 and PB-06-003 were similar.

**Table 6: Common Adverse Events (>5% of treated patients)**

| System Organ Class | Preferred Term | 30 U/kg group N=16 | 60 U/kg group N=16 | All treatment groups N=32 |
|---|---|---|---|---|
| **Infections and Infestations** | | | | |
| | URI/Cold | 3 (19%) | 4 (25%) | 7 (22%) |
| | Pharyngitis/Throat infection | 4 (25%) | 2 (13%) | 6 (19%) |
| | Eye infection | 1 (6%) | 1 (6%) | 2 (6%) |
| | Influenza/Flu | 1 (6%) | 3 (19%) | 4 (13%) |
| | UTI/Pyelonephritis | 1 (6%) | 2 (13%) | 3 (9%) |
| | Gastroenteritis | 1 (6%) | 1 (6%) | 2 (6%) |
| | Otitis externa | 1 (6%) | 2 (13%) | 2 (6%) |
| **Musculoskeletal and Connective Tissue Disorders** | | | | |
| | Arthralgia/back pain | 1(6%) | 3 (19% | 4 13%) |
| **Nervous System Disorders** | | | | |
| | Headache | 1 (6%) | 5 (32%) | 6 (19%) |
| | Dizziness | 2 (13%) | 1 (6%) | 3 (9%) |
| **Gastrointestinal Disorders** | | | | |
| | Nausea | 1 (6%) | 2 (13%) | 3 (9%) |
| | Vomiting | 2 (13%) | 0 (0%) | 2 (6%) |
| | Abdominal pain | 2 (13%) | 0 (0%) | 2 (6%) |
| **General Disorders and Administration Site Conditions** | | | | |
| | Fatigue/tiredness | 1 (6%) | 2 (13%) | 3 (9%) |
| | Warmth | 1 (6%) | 1 (6%) | 22 (6%) |
| **Skin and Subcutaneous Tissue Disorders** | | | | |
| | Itching | 1 (6%) | 1 (6%) | 2 (6%) |
| | Skin mycosis | 1 (6%) | 1 (6%) | 2 (6%) |
| **Vascular disorders** | | | | |
| | Facial flushing | 1 (6%) | 1 (6%) | 2 (6%) |
| | Hypertension/high BP | 2 (13%) | 0 (0%) | 2 (6%) |
| **Blood and Lymphatic System Disorders** | | | | |
| | Enlarged lymph nodes | 1 (6%) | 1 (6%) | 2 (6%) |
| **Immune System Disorders** | | | | |
| | Hypersensitivity | 1* (6%) | 1* (6%) | 2 (6%) |
| **Respiratory, Thoracic, and Mediastinal Disorders** | | | | |
| | Epistaxis | 2 (13%) | 0 (0%) | 2 (6%) |

*Patient was discontinued from the trial
(copied from clinical review, C. Epps)

The clinical reviewer evaluated the incidence of abnormal laboratory findings, abnormal vital sign reports, and abnormal ECG and echocardiogram reports. There was no pattern or apparent safety signal uncovered with any of these parameters.

The effect of immunogenicity is also an important concern for all ERTs. The applicant reported that only two patients developed anti-taliglucerase IgG antibodies in Study PB-06-001. As noted above (section 8: Clinical/Statistical: Efficacy; Immunogenicity), the antibody assay cut-off that the applicant used was not acceptable by the immunogenicity reviewer. Therefore, it was not possible to fully assess the effect of immunogenicity of the safety of the

product during this review cycle. Of two patients that developed IgG antibodies to taliglucerase as reported by the applicant, neither of the patients developed anaphylaxis, allergic reactions, or immune-mediated reactions. A complete review of the effect of immunogenicity must be re-evaluated after the applicant provides an acceptable antibody assay.

Conclusions and Recommendations

Overall, the clinical reviewer concluded that the size of the safety database was acceptable because of the rarity of the disease. I agree with the reviewer's assessment. However, there were very few patients in the safety database with data on longer-term treatment (i.e., more than 12 months). Immune-mediated reactions and chronic allergic reactions may be seen in ERTs with continued long-term treatment. Therefore, I would recommend that additional longer-term safety data from study PB-06-003 should be required as part of the Complete Response. Additionally, I would recommend that a complete study report for Study PB-06-002 be required as part of the Complete Response to assess safety in patients who switched from imiglucerase to taliglucerase more fully.

Additionally, the impact of immunogenicity could not be fully assessed during this review cycle because the applicant did not provide an adequate cut-point for the confirmatory immunodepletion assay for anti-taliglucerase alfa antibody formation. Therefore, I recommend that an acceptable cut-point for the confirmatory immunodepletion assay for anti-taliglucerase alfa antibodies be required as a part of the Complete Response, and that the applicant must reanalyze all available safety data based on the revised assay.

Postmarketing safety requirements were not negotiated because the safety data could not be completed reviewed during this review cycle.

Overall recommendations

Based on the overall review of the safety and efficacy of taliglucerase alfa, significant questions remain unanswered. Therefore, I recommend that a Complete Response action be taken for this submission. The following comments will be provided to the applicant in the complete response letter, and represent deficiencies that must be addressed in their complete response.

1. The immunogenic potential of taliglucerase alfa and its impact on efficacy and safety cannot be adequately evaluated.
    a. Propose an acceptable cut-point for your confirmatory anti-product IgG antibody assay and submit a re-analysis of the impact of anti-product antibody development on the efficacy and safety of taliglucerase alfa.
    b. Develop an acceptable neutralizing antibody assay and submit a re-analysis of the impact of neutralizing antibody development on the efficacy and safety of taliglucerase alfa.
2. There are insufficient data provided to assess the efficacy and safety of taliglucerase alfa in patients switched from other enzyme replacement therapies. Submit the final

study report from PB-06-002, and a minimum of 12 months of efficacy and safety data from PB-06-003 for patients switched from other enzyme replacement therapies to taliglucerase alfa.

3. Longer-term safety data were insufficient to evaluate the chronic immune-mediated adverse events that are typically associated with enzyme replacement therapies, and Gaucher disease-specific bone events. Provide additional long-term safety data from PB-06-003.

# 10. Advisory Committee Meeting

This product is considered a new molecular entity. However, it shares similarity with previously approved ERTs for Gaucher disease; Ceredase, Cerezyme, and Velaglucerase. Additionally, no new or unique concerns were identified during the review of this product compared to other drugs in this class. Therefore, no advisory committee meeting convened for this product during this review cycle.

# 11. Pediatrics

Taliglucerase alfa received orphan designation on September 3, 2009. Therefore, the regulations that pertain to the Pediatric Equity in Research Act (PREA) do not apply to taliglucerase alfa. The submission was not presented to the Pediatric Review Committee (PeRC).

# 12. Other Relevant Regulatory Issues

**A. DSI audits and Financial Disclosures**

All of the study sites for the single pivotal trial were conducted at foreign sites. The Division of Scientific Investigations (DSI) performed clinical site inspections at two foreign sites (Site #30, Clinical Center of Serbia, Belgrade, Serbia and Site #10, Shaare Zedek Medical Center, Jerusalem, Israel). The DSI inspector found that the data from the two clinical study sites inspected are reliable and can be used in support of the BLA. Additionally, an inspection of the applicant, Protalix Biotherapeutics was performed by DSI because taliglucerase is a new molecular entity. The inspection of the applicant revealed no significant deficiencies and the DSI inspector noted that the applicant adequately monitored the studies conducted in the application.

Financial disclosures were submitted by the applicant for one investigator, [redacted] This site underwent DSI audit (see above) and the inspection did not note any

significant deficiencies. Thus, despite the significant financial relationships between the [redacted] and the applicant, the conduct of the studies does not appear to have been affected by these financial arrangements.

**B. Clinical Consults**

There were no clinical consults obtained for the current review.

**C. Drug shortage**

There are currently two approved ERT products for Type 1 Gaucher disease in the U.S.; Cerezyme (imiglucerase) and VPRIV (velaglucerase). U.S. production of Cerezyme was temporarily suspended in June 2009 due to manufacturing issues at Genzyme's Allston Landing facility, where Cerezyme is manufactured. Although Velaglucerase alfa was made available through a treatment protocol in July 2009 and received U.S. approval in February 2010, there was an on-going drug shortage at the time of submission of this application. Genzyme issued a statement on January 11, 2011 that the supply of Cerezyme had been restored for all patients currently receiving therapy and that Cerezyme will be available to new Gaucher patients. We have not received any reports of any U.S. Gaucher patients that are unable to access ERT at the present time. Therefore, it appears that the drug shortage for U.S. Gaucher patients has resolved at the time of this review.

# 13. Labeling

Proprietary name

During this review cycle, the originally proposed trade name of [redacted] was submitted for review. A review of the trade name was performed by Z. Oleszczuk in the Division of Medication Errors Prevention and Analysis (DMEPA). The proposed name, [redacted] was rejected by DMEPA [redacted] The applicant subsequently submitted a new proposed name, Elelyso. A review of this trade name was performed by Z. Oleszczuk, DMEPA. The trade name, Elelyso, was found to be acceptable. However, this proposed name must be reviewed again with the applicant's complete response submission.

Physician labeling/ Carton and immediate container labels

Final product labeling, as well as carton and container labeling were not satisfactorily negotiated during the current review cycle because deficiencies in the submission leading to a Complete Response action precluded a complete review and negotiation of final labeling with the applicant. The applicant will be required to submit proposed physician labeling and carton and container labeling with their Complete Response.

Patient labeling/Medication guide (if considered or required)

A final determination of the requirement for patient labeling and/or medication guide was not made during this review cycle. However, other approved products in this class have not included a medication guide because ERTs are intended to be administered by specialized infusion staff. Taliglucerase, like other ERTs for Gaucher disease would not be directly supplied to the patient. Therefore, it is unlikely that a medication guide will be required.

# 14. Recommendations/Risk Benefit Assessment

Recommended Regulatory Action
The current application contains deficiencies that have not been satisfactorily addressed. These include serious CMC, immunogenicity, product quality microbiology, clinical pharmacology, and clinical issues that have not been resolved. Therefore, I recommend that a Complete Response (CR) action be taken for this application.

Risk Benefit Assessment
The benefit of taliglucerase alfa in the treatment of Gaucher disease appears to have been demonstrated based on a single study. However, the relative benefit of the product compared to two products already approved in the same class of biologic products to treat Gaucher disease has not yet been clearly demonstrated. Furthermore, the risk of immunogenicity for this product could not be evaluated during this review cycle because the immunogenicity assays used by the applicant to determine the immunogenicity of the product were not acceptable. Finally, it should be noted that at the present time, the drug shortage for Gaucher patients appears to have eased. Genzyme reported in January 2011 that Cerezyme supplies were improved and that new Gaucher patients would be able to access the product. Additionally, VPRIV has been available since February 2010 and we have not received any reports of any U.S. Gaucher patients that are unable to access ERT at the present time. It appears that the drug shortage for U.S. Gaucher patients has resolved at the time of this review. Therefore, the risk benefit assessment is currently unacceptable and precludes approval of the product during this review cycle.

Additionally, the clinical and statistical reviewer both recommended that an additional study must be performed to establish the relative efficacy and safety of taliglucerase alfa compared to other approved ERT products because the current submission contained inadequate information to assess this. I agree that additional information must be included in the resubmission to address this deficiency. However, I do not agree that an additional clinical study must be performed as a condition for resubmission. I recommend that data from study PB-06-002 and PB-06-003 be carefully evaluated to assess the clinical outcome of patients switched from Cerezyme to taliglucerase alfa who have received treatment for at least 12 months. If these data are sufficient to demonstrate the relative effectiveness of taliglucerase alfa compared to Cerezyme, then an additional study may not be necessary. This was discussed with both the clinical and statistical reviewer who both agree that this recommendation was reasonable. However, the reviewers continue to express concern that the data contained in PB-06-002 and PB-06-003 may not sufficient, and if not, they agree that an additional clinical study should be required at that time.

Recommendation for Postmarketing Risk Evaluation and Management Strategies
Postmarketing risk management activities were not reviewed extensively during this review cycle because a Complete Response action is recommended. However, during the review of the application, no special issues that would require postmarketing risk management activities were identified. Therefore, the Complete Response action will not include recommended risk management strategies to be included in a Complete Response.

Recommendation for other Postmarketing Requirements and Commitments
Postmarketing requirements and commitments were not reviewed extensively during this review cycle because a Complete Response action is recommended. Therefore, the Complete Response action will not include recommended specific recommendations for postmarketing requirements and commitments to be included in a Complete Response.

Recommended Comments to Applicant

**Clinical**
The following recommended comments regarding further clinical information to be included in the complete response are as follows:

1. The immunogenic potential of taliglucerase alfa and its impact on efficacy and safety cannot be adequately evaluated.

    a. Propose an acceptable cut-point for your confirmatory anti-product IgG antibody assay and submit a re-analysis of the impact of anti-product antibody development on the efficacy and safety of taliglucerase alfa.
    b. Develop an acceptable neutralizing antibody assay and submit a re-analysis of the impact of neutralizing antibody development on the efficacy and safety of taliglucerase alfa.

2. There are insufficient data provided to assess the efficacy and safety of taliglucerase alfa in patients switched from other enzyme replacement therapies. Submit the final study report from PB-06-002, and a minimum of 12 months of efficacy and safety data from PB-06-003 for patients switched from other enzyme replacement therapies to taliglucerase alfa.

3. Longer-term safety data were insufficient to evaluate the chronic immune-mediated adverse events that are typically associated with enzyme replacement therapies, and Gaucher disease-specific bone events. Provide additional long-term safety data from PB-06-003.

**Clinical Pharmacology**
The following recommended comments regarding further clinical pharmacology information to be included in the complete response are as follows:

4. The immunogenic potential of taliglucerase alfa and its impact on the pharmacokinetic and pharmacodynamic (PK and PD) parameters cannot be adequately evaluated.

    a. Propose an acceptable confirmatory cut-point for your anti-product IgG antibody assay and submit a re-analysis of the impact on PK and PD parameters in patients treated with taliglucerase alfa.
    b. Develop an acceptable neutralizing antibody assay and submit a re-analysis of the impact on PK and PD parameters in patients treated with taliglucerase alfa.

**Product Quality**
The following recommended comments regarding further product quality information to be included in the complete response are as follows:

<u>Specifications and Assay Validation</u>

5. Results of USP <788> particulate testing and appearance testing on reconstituted drug product have not been submitted to the NDA. Both tests provide a useful measure of product quality that is not monitored by other tests you have proposed. Add these tests to the release and stability specifications and provide available results for release and stability testing of the three conformance lots and any additional results you may have.

6. A potency assay that quantitatively measures specific receptor binding and/or high affinity internalization into cells is required since internalization is a critical component of taliglucerase alfa's mechanism of action and it is not fully assessed in your current potency assay. The assay should use multiple taliglucerase alfa concentrations to generate a complete dose-response curve in order to calculate the half-maximal effective concentration ($EC_{50app}$). Develop and implement this assay for use in release and stability testing.

7. Some SE-HPLC chromatographs exhibit a [redacted]. Because this [redacted] may reflect variability in a product-related variant, it should be identified and, if necessary, controlled. Characterize the protein [redacted] and determine whether a control strategy that better monitors this product attribute(s) should be implemented. Provide the results of your analyses and any proposed changes to your specifications.

8. RP-HPLC chromatograms suggest that taliglucerase alfa variants [redacted]. The risk to product quality is expected to vary depending on the nature of the variant. Thus, in order to establish an appropriate control strategy, you should identify and control for the quantity of these variants, if present. It may be useful to alter assay conditions or gradients to [redacted]. Provide information on the presence of unresolved variants and, if present, provide a revised specification that more accurately quantitates and controls these variants together with supporting data.

9. Enzyme kinetic parameters and specific activity are measured using synthetic p-nitrophenyl-glucopyranoside (pNP-Glc) substrate. [redacted] may be less sensitive in detecting changes to product quality. Provide enzyme kinetic data to determine the enzyme kinetic parameters, Km and kcat [redacted] Include a detailed description of the assay, supporting assay qualification data, as well as a justification for why this test should not be added to the release and stability specifications.

10. Stability testing of diluted drug product in infusion bags did not include USP <788> particulate testing or information on the impact of dilution on subvisible particulates that are between [redacted]. USP <788> testing results are critical to mitigate the risk associated with occlusion of small blood vessels and small subvisible particles may pose an immunogenicity risk. Provide USP <788> particulate testing data for in-use stability studies and an analysis of particulates between [redacted].

11. The mannose content specification is based on a MALDI-TOF analysis of taliglucerase alfa. However, the property that is being measured in the MALDI-TOF analysis is mass to charge ratio, not mannose content. Thus, the acceptance criterion should be set around the mass to charge ratio and the mannose content acceptance criterion should be removed from the MALDI-TOF specification. Provide the new specification together with supporting data.

12. The acceptance criterion for moisture content in drug product is [redacted] for both release and stability testing. Release and stability testing results consistently show moisture content to be below [redacted] and no data were submitted indicating that a [redacted] moisture content would not have an adverse impact on product stability throughout the product's dating period. Amend the moisture content acceptance criterion to reflect your manufacturing capability and consideration of any additional knowledge you may have concerning the impact of moisture on product stability and provide the new specification, if appropriate, together with supporting data.

13. Monosaccharide content and glycan structure analysis submitted in the characterization section of the NDA contained inconsistent results. Monosaccharide content analysis on two batches indicated that the [redacted] whereas the glycan analysis data determined that [redacted] of the glycan structures have a [redacted] Provide an explanation for these results or submit data that identify the more accurate analysis using batches made in [redacted]

14. The acceptance criteria for the enzyme kinetic parameters $K_m$ and $V_{max}$ are [redacted] respectively. An analysis of 40 drug substance batches resulted in mean and standard deviations for $K_m$ and $V_{max}$ equal to [redacted] respectively. Consequently, the acceptance criteria appear too wide and should be amended to reflect process capability and clinical experience. Provide the revised specification for enzyme kinetic parameters or your justification as to why your proposal ensures reproducible product potency.

15. In a [redacted] vial drug product fill, the sampling plan calls for [redacted] to be collected for moisture content testing. [redacted] vials are tested and the mean value is reported on the certificate of analysis. Because the moisture content in an individual vial will vary within any given lot, the proposed sampling plan should provide a reasonable assessment of the variability of the results within a lot. While data from a robust validation study will

provide a basis for establishing the sampling plan for the moisture specification, your current sample size and the mean value set as the reportable result are insufficient to assess the moisture content of the final drug product. Submit the revised specification for moisture content with these considerations in mind and provide a justification for your proposal.

16. Chromatograms for drug substance and drug product RP-HPLC analyses contain data from [redacted]. Perform the RP-HPLC analysis such that data from [redacted] and from [redacted] is included so that all potential impurities and contaminants can be detected and controlled if necessary. Provide chromatograms where all data are shown [redacted] minutes) on lots evaluated in the [redacted] comparability studies.

17. The isoelectric focusing (IEF) assay has acceptance criteria of [redacted] in a pI range of [redacted] reportedly because of assay variation. This level of assay variability is not consistent with the expected validation characteristics for this type of assay. Develop, implement, and provide data on a validated IEF method in which the reference standard always produces the same number of bands in a consistent pI range. In addition, each gel should have a quantity of reference standard loaded near the limits of detection to verify the sensitivity of the analysis.

18. The [redacted] assay results are rounded off to the nearest integer which can mask significant differences in [redacted] between lots. Report all [redacted] assay results to two significant digits without rounding off to the nearest integer, revise the acceptance criterion accordingly and submit the revised specification.

19. [redacted]

20. The peptide map specification calls for [redacted] peptide peaks where a countable peak is defined as [redacted] Justify the use of this acceptance criterion in light of the potential amounts of impurities and contaminants that would be acceptable, or revise the criteria for countable peaks. Also, include a revision of the acceptance criteria such that relative peak areas on several selected peptides are specified. Provide the new specification together with supporting data.

21. A host cell protein standard curve is used to determine the levels of host cell proteins in the drug substance. The data from the standard curve is fit to a four parameter logistic regression model even though the data do not reach a plateau and the fitted curve is not

fully determined. However, there is a simple linear relationship between host cell protein and assay response. Provide a justification for the use of a four parameter logistic regression model or use a linear regression model to generate a host cell protein standard curve. Submit the revised specification along with the supporting analytical method validation data.

Comparability

22. The relative amounts of the individual glycans in the glycan profile shifted upon the switch to [redacted]. Since the glycan structures are critical to taliglucerase alfa's mechanism of action, a change in the concentration of the glycan structures has the potential to adversely impact clinical performance. Using a potency assay that quantitatively measures specific receptor binding and/or high affinity internalization into cells (see previous comment), perform a head-to-head comparison of three drug substance lots of taliglucerase alfa manufactured in [redacted]

23. Results for SE-HPLC data provided in the NDA are reported as [redacted]. As [redacted] may represent a different risk to product quality, they should be independently monitored and controlled. To support your revised acceptance criteria, provide all SE-HPLC data available to date in the application with [redacted] reported separately. For comparison purposes, provide tabulated drug product stability SE-HPLC data separating drug product lots that were manufactured with drug substance made exclusively in [redacted]

24. Your SE-HPLC test method employed a UV detector. However, use of a light scattering detector may allow [redacted] that migrate in the void volume to be observed following SE-HPLC. This provides a much more sensitive qualitative method for monitoring this product attribute. Perform a head-to-head comparison of three drug product lots manufactured exclusively from drug substance made in [redacted] using light scatter detection and provide the results in your resubmission.

Process Validation

25. The time limits for individual manufacturing steps and for the complete manufacturing process are not clearly defined in the NDA. For example, strict limits for [redacted] Provide this information and relate it to the processes used to manufacture clinical study lot PB-06-001, commercial validation lots and the genomic stability sequencing study.

26. Process validation reports indicate that vials containing drug product were put on [redacted] lyophilizer shelves. Validation of the lyophilization process should

include assessment of vials [redacted] and in different positions within a shelf to confirm consistency of the lyophilization process. Provide a revised validation protocol and report including the results for moisture content testing.

27. [redacted]

Control of Impurities

28. The testing to demonstrate that the master cell bank was free of plant specific viruses tabulated the results without providing data on the suitability of the PCR methods to detect viruses. In order to interpret the results you provided, the suitability of methods for their intended purpose needs to be assessed. Provide the assay qualification data and a description of the system suitability controls for each PCR method used to detect plant specific viruses.

29. The compound [redacted] is a component [redacted] levels in drug substance or drug product were not determined. [redacted] may exhibit toxicity to humans [redacted] and is therefore viewed as a [redacted] impurity that should be well controlled. Provide a control strategy to either include a limit on [redacted] to a level that will not impact product quality as it may relate to safety or efficacy, or validate that the process can clear [redacted] to an appropriate level.

30. [redacted], but its final concentration in drug product has not been determined. The label should accurately describe the final concentration of all excipients which should be confirmed at release. Provide the results on the [redacted] concentration for three drug product lots and provide your justification for not implementing the determination of [redacted] as a drug product release test.

**Product Quality Immunogenicity**
The following recommended comments regarding further immunogenicity information to be included in the complete response are as follows:

31. The concentration of rabbit anti taliglucerase alfa IgG antibodies [redacted]) that you used for the positive control-1 (PC-1) for the anti-product IgG assay quality assessment (binding assay) was high. The agency recognizes that the limit of detection may be different due to affinity differences of the antibodies in the assay. However, in order to ensure reliable performance of the assay, a lower concentration for the positive control that will produce a signal close to the established cut-point of the assay should also be used. Confirm that your assay contains a low concentration positive control that can reproducibly produce a response closer to the established cut-point of your assay.

32. You set the cut-point at [redacted] for the immunodepletion assay to confirm the antibody status of patients. The agency recommends that the confirmatory cut-point be set based on assay precision. Re-establish the immunodepletion assay cut-point based on assay precision using serum from healthy human subjects and from treatment-naïve patients, if available.

33. In your drug tolerance study, you used control antibodies at a concentration of [redacted] to assess drug tolerance. Your assay is insufficient to address drug tolerance at low concentrations of anti-product antibodies. Repeat your drug tolerance study in the presence of low concentrations of control antibodies.

34. Develop appropriate quality controls in the neutralizing antibody assay and establish acceptance criteria based on these controls.

35. The specificity assessment should be designed to show that the drug product specifically binds to the antibodies induced by the product in human serum in the presence of exogenously added interfering molecules of similar size and charge (e.g., inclusion of IgG in IgE assay development).

36. We recognize that an alternative control for the anti-product IgE antibody assay may be required if a human positive control is not available, and that the detection limit may vary depending on antibody affinity. However, an estimation of assay sensitivity expressed in mass units is necessary to ensure assay suitability and performance for the intended purpose. Determine assay sensitivity and report the results.

**Product Quality Microbiology**
The following recommended comments regarding further product quality microbiology information to be included in the complete response are as follows:

37. With regard to the validation of [redacted] provide a bioburden data summary to justify this [redacted].

38. For the [redacted] Lyophilizer, provide summary data from three consecutive successful [redacted] runs with acceptable [redacted] results. [redacted]

39. Provide validation summary reports for sterility and bacterial endotoxin test methods.

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/s/

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LYNNE P YAO
02/24/2011

# EXHIBIT F

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration
Silver Spring MD 20993

NDA 22-458

**NDA ACKNOWLEDGMENT**

Protalix Ltd.
c/o Target Health Inc.
Attention: Glen D. Park, Pharm D.
261 Madison Avenue
24th Floor
New York, NY 10016

Dear Dr. Park:

We have received your New Drug Application (NDA) submitted under section 505(b) of the Federal Food, Drug, and Cosmetic Act (FDCA) for the following:

Name of Drug Product: [redacted](taliglucerase alfa) for injection, 200 units

Date of Application: April 26, 2010

Date of Receipt: April 26, 2010

Our Reference Number: NDA 22-458

Unless we notify you within 60 days of the receipt date that the application is not sufficiently complete to permit a substantive review, we will file the application on June 25, 2010, in accordance with 21 CFR 314.101(a).

If you have not already done so, promptly submit the content of labeling [21 CFR 314.50(l)(1)(i)] in structured product labeling (SPL) format as described at http://www.fda.gov/ForIndustry/DataStandards/StructuredProductLabeling/default.htm. Failure to submit the content of labeling in SPL format may result in a refusal-to-file action under 21 CFR 314.101(d)(3). The content of labeling must conform to the content and format requirements of revised 21 CFR 201.56-57.

The NDA number provided above should be cited at the top of the first page of all submissions to this application. Send all submissions, electronic or paper, including those sent by overnight mail or courier, to the following address:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Gastroenterology Products
5901-B Ammendale Road
Beltsville, MD 20705-1266

All regulatory documents submitted in paper should be three-hole punched on the left side of the page and bound. The left margin should be at least three-fourths of an inch to assure text is not obscured in the fastened area. Standard paper size (8-1/2 by 11 inches) should be used; however, it may occasionally be necessary to use individual pages larger than standard paper size. Non-standard, large pages should be folded and mounted to allow the page to be opened for review without disassembling the jacket and refolded without damage when the volume is shelved. Shipping unbound documents may result in the loss of portions of the submission or an unnecessary delay in processing which could have an adverse impact on the review of the submission. For additional information, please see http://www.fda.gov/Drugs/DevelopmentApprovalProcess/FormsSubmissionRequirements/DrugMasterFilesDMFs/ucm073080.htm

If you have any questions, call me at (301) 796-2259.

Sincerely,

*{See appended electronic signature page}*

Chantal Phillips, M.S.H.S.
CDR, U.S. Public Health Service
Regulatory Health Project Manager
Division of Gastroenterology Products
Office of Drug Evaluation III
Center for Drug Evaluation and Research

| Application Type/Number | Submission Type/Number | Submitter Name | Product Name |
|---|---|---|---|
| NDA-22458 | ORIG-1 | PROTALIX LTD | PLANT CELL EXPRESSED RECOMBINANT HUMAN G |

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/s/

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CHANTAL N PHILLIPS
04/28/2010

# EXHIBIT G

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration
Silver Spring MD 20993

NDA 022458

**NDA APPROVAL**

Protalix Ltd.
c/o Target Health Inc.
261 Madison Avenue, 24th Floor
New York, NY 10016

Attention: Glen D. Park, Pharm.D.
Senior Director, Clinical/Regulatory Affairs

Dear Dr. Park:

Please refer to your New Drug Application (NDA) dated April 26, 2010, received April 26, 2010, submitted under section 505(b) of the Federal Food, Drug, and Cosmetic Act for ELELYSO (taliglucerase alfa) for injection.

We acknowledge receipt of your amendments dated April 30, 2010; May 4, 2010; June 7(2), 11, 18, and 30, 2010; July 21, 2010; August 3 and 20, 2010; September 10 and 27, 2010; October 1, 2010; November 24 and 30, 2010; December 2, 3, 10, 20, 21, 23, 27 and 28, 2010; August 1 and 8, 2011; October 5 and 7, 2011; November 1 and 15(2), 2011; December 13, 2011; January 20 and 23, 2012; February 10, 2012; March 1, 22, and 28, 2012; and April 2, 11, 18, 19, 20, 25(2), and 27, 2012.

The August 1, 2011, submission constituted a complete response to our February 24, 2011, action letter.

This new drug application provides for the use of ELELYSO (taliglucerase alfa) for injection for use as a long-term enzyme replacement therapy in patients with Type 1 Gaucher disease.

We have completed our review of this application, as amended. It is approved, effective on the date of this letter, for use as recommended in the agreed-upon labeling text and with the minor editorial revisions listed below for the carton and container.

1. Remove the statements, [redacted] (b)(4) to comply with 21 CFR 201.51 and the U.S. Pharmacopeia 10/1/10-2/1/11, USP 33/NF 28. An overfill justification is provided in section 3.2.P.2.2.

2. Please revise the statement, [redacted] (b)(4) to "Each vial contains taliglucerase alfa 200 units".

EXHIBIT
G
PENGAD 800-631-6989

## CONTENT OF LABELING

As soon as possible, but no later than 14 days from the date of this letter, submit the content of labeling [21 CFR 314.50(l)] in structured product labeling (SPL) format using the FDA automated drug registration and listing system (eLIST), as described at http://www.fda.gov/ForIndustry/DataStandards/StructuredProductLabeling/default.htm. Content of labeling must be identical to the enclosed labeling (text for the package insert). Information on submitting SPL files using eLIST may be found in the guidance for industry titled "SPL Standard for Content of Labeling Technical Qs and As" at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072392.pdf.

The SPL will be accessible via publicly available labeling repositories.

## CARTON AND IMMEDIATE CONTAINER LABELS

Submit final printed carton and container labels that are identical to the submitted carton and immediate container labels, except with the revisions listed above, as soon as they are available, but no more than 30 days after they are printed. Please submit these labels electronically according to the guidance for industry titled "Providing Regulatory Submissions in Electronic Format – Human Pharmaceutical Product Applications and Related Submissions Using the eCTD Specifications (June 2008)." Alternatively, you may submit 12 paper copies, with 6 of the copies individually mounted on heavy-weight paper or similar material. For administrative purposes, designate this submission "**Final Printed Carton and Container Labels for approved NDA 022458**." Approval of this submission by FDA is not required before the labeling is used.

Marketing the product with FPL that is not identical to the approved labeling text may render the product misbranded and an unapproved new drug.

## ADVISORY COMMITTEE

Your application for ELELYSO (taliglucerase alfa) for injection was not referred to an FDA advisory committee because the application did not raise significant safety or efficacy issues that were unexpected for a drug in this class.

## REQUIRED PEDIATRIC ASSESSMENTS

Under the Pediatric Research Equity Act (PREA) (21 U.S.C. 355c), all applications for new active ingredients, new indications, new dosage forms, new dosing regimens, or new routes of administration are required to contain an assessment of the safety and effectiveness of the product for the claimed indication(s) in pediatric patients unless this requirement is waived, deferred, or inapplicable.

Because this drug product for this indication has an orphan drug designation, you are exempt from this requirement.

## POSTMARKETING REQUIREMENTS UNDER 505(o)

Section 505(o)(3) of the Federal Food, Drug, and Cosmetic Act (FDCA) authorizes FDA to require holders of approved drug and biological product applications to conduct postmarketing studies and clinical trials for certain purposes, if FDA makes certain findings required by the statute.

We have determined that an analysis of spontaneous postmarketing adverse events reported under subsection 505(k)(1) of the FDCA will not be sufficient to assess known serious risks of allergic and immune-mediated reactions or to identify unexpected serious risks related to the development of neutralizing anti-drug antibodies or plant-specific sugar antibodies and cellular uptake inhibition in adult and pediatric patients with Type 1 Gaucher disease treated with ELELYSO (taliglucerase alfa) for injection, or to identify unexpected serious adverse effects on 1) pregnancy outcomes, 2) fetal outcomes (teratogenicity), or 3) outcomes in newborns and infants exposed to ELELYSO (taliglucerase alfa) for injection and through breast-feeding.

Furthermore, the new pharmacovigilance system that FDA is required to establish under section 505(k)(3) of the FDCA will not be sufficient to assess this serious risk.

Therefore, based on appropriate scientific data, FDA has determined that you are required to conduct the following:

1895-1 To develop a validated, sensitive, and accurate assay for the detection of neutralizing antibodies to ELELYSO (taliglucerase alfa) for injection that is expected to be present in the serum at the time of patient sampling. A summary of the validation exercise including supporting data, a summary of the development data supporting assay suitability for parameters not assessed in the validation exercise, and the assay SOP will be provided to FDA.

The timetable you agreed to on April 26, 2012, states that you will conduct this study according to the following schedule:

| | |
|---|---|
| Final Protocol Submission: | 09/2012 |
| Study Completion: | 03/2013 |
| Final Report Submission: | 07/2013 |

1895-2 To develop a validated, sensitive, and accurate assay for the assessment of cellular uptake inhibition by cell surface mannose receptors due to the presence of neutralizing antibodies to ELELYSO (taliglucerase alfa) for injection that is expected to be present in the serum at the time of patient sampling. A summary of the validation exercise including supporting data, a summary of the development data supporting assay suitability for parameters not assessed in the validation exercise, and the assay SOP will be provided to FDA.

The timetable you agreed to on April 26, 2012, states that you will conduct this study according to the following schedule:

| | |
|---|---|
| Final Protocol Submission: | 12/2012 |
| Study Completion: | 06/2013 |
| Final Report Submission: | 10/2013 |

1895-3 To develop a validated, sensitive, and accurate assay for the detection of antibodies to plant-specific sugars in ELELYSO (taliglucerase alfa) for injection that is expected to be present in the serum at the time of patient sampling. A summary of the validation exercise including supporting data, a summary of the development data supporting assay suitability for parameters not assessed in the validation exercise, and the assay SOP will be provided to FDA.

The timetable you agreed to on April 26, 2012, states that you will conduct this study according to the following schedule:

| | |
|---|---|
| Final Protocol Submission: | 12/2012 |
| Study Completion: | 06/2013 |
| Final Report Submission: | 10/2013 |

1895-4 To conduct an assessment of neutralizing anti-drug antibody (ADA) response and presence of antibodies against plant-specific sugars in ELELYSO (taliglucerase alfa) for injection in patient plasma samples. Validated assays (developed under 1895-1, 1895-2 and 1895-3) capable of sensitively detecting neutralizing ADA responses and antibodies to plant-specific sugars that are expected to be present at the time of patient sampling will be used. The neutralizing ADA response, cellular uptake inhibition and the presence of plant-specific sugar antibodies will be evaluated in all archived sampling time points available from all patients in Phase 3 trials (PB-06-001, PB-06-002, PB-06-003, and PB-06-005). Analysis will evaluate immunogenicity rates and individual patient titers to assess the impact of neutralizing antibody levels, cellular uptake inhibition, and plant-specific sugar antibody levels on parameters of safety as well as on the pharmacokinetics (PK), pharmacodynamics (PD), and efficacy of ELELYSO (taliglucerase alfa) for injection where data are available.

The timetable you agreed to on April 26, 2012, states that you will conduct this study according to the following schedule:

| | |
|---|---|
| Final Protocol Submission: | 12/2012 |
| Study Completion: | 11/2013 |
| Final Report Submission: | 03/2014 |

1895-5 To evaluate the long-term safety and efficacy of ELELYSO (taliglucerase alfa) for injection in a registry of Gaucher disease patients being treated with ELELYSO (taliglucerase alfa) for injection. Detailed clinical status information will be collected at study entry and on an annual basis for 10 years. An interim report will be submitted after completion of the first 5 years of the study.

The timetable you agreed to on April 30, 2012, states that you will conduct this study according to the following schedule:

| | |
|---|---|
| Final Protocol Submission: | 06/2013 |
| Interim Report Submission: | 07/2019 |
| Study Completion: | 10/2023 |
| Final Report Submission: | 07/2024 |

1895-6 To evaluate the effect of ELELYSO (taliglucerase alfa) for injection on pregnancy and fetal outcomes, and to collect detailed clinical status information on newborns and infants whose mothers are treated with ELELYSO (taliglucerase alfa) for injection during lactation. This study may be completed as a sub-study within the registry (1895-5). An interim report will be submitted after completion of the first 5 years of the study.

The timetable you agreed to on April 30, 2012, states that you will conduct this study according to the following schedule:

| | |
|---|---|
| Final Protocol Submission: | 06/2013 |
| Interim Report Submission: | 06/2019 |
| Study Completion: | 10/2023 |
| Final Report Submission: | 07/2024 |

Finally, we have determined that only a clinical trial (rather than a nonclinical or observational study) will be sufficient to assess known serious risks of allergic and immune-mediated reactions or to identify unexpected serious risks related to the development of neutralizing anti-drug antibodies or plant-specific sugar antibodies and cellular uptake inhibition in adults and pediatric patients with Type 1 Gaucher disease treated with ELELYSO (taliglucerase alfa) for injection.

Therefore, based on appropriate scientific data, FDA has determined that you are required to conduct the following:

1895-7 To complete the ongoing trial PB-06-005, entitled "A Multicenter, Double-blind, Randomized Safety and Efficacy Study of Two Dose Levels of Taliglucerase Alfa in Pediatric Subjects with Gaucher Disease." This trial will obtain safety and efficacy data in pediatric patients with Type 1 Gaucher disease, including data on allergic and immune-mediated reactions, and unexpected risks from antibody development. The trial was initiated in October 2010.

The timetable you agreed to on April 26, 2012, states that you will conduct this trial according to the following schedule:

| | |
|---|---|
| Trial Completion: | 06/2012 |
| Final Report Submission: | 09/2012 |

1895-8 To complete the ongoing trial PB-06-002, entitled "A Multicenter, Open-label, Switchover Trial to Assess the Safety and Efficacy of Taliglucerase alfa in Patients with Gaucher Disease Treated with Imiglucerase (Cerezyme®) Enzyme Replacement Therapy." This trial will obtain safety and efficacy data in adult and pediatric patients with Type 1 Gaucher disease, including data on allergic and immune-mediated reactions, and unexpected risks from antibody development. The trial was initiated in the U.S. in April 2009.

The timetable you agreed to on April 26, 2012, states that you will conduct this trial according to the following schedule:

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|---|---|
| Trial Completion: | 03/2013 |
| Final Report Submission: | 06/2013 |

Submit the protocol(s) to your IND 069703, with a cross-reference letter to this NDA. Submit all final report(s) to your NDA. Prominently identify the submission with the following wording in bold capital letters at the top of the first page of the submission, as appropriate: **"Required Postmarketing Protocol Under 505(o)", "Required Postmarketing Final Report Under 505(o)", "Required Postmarketing Correspondence Under 505(o)"**.

Section 505(o)(3)(E)(ii) of the FDCA requires you to report periodically on the status of any study or clinical trial required under this section. This section also requires you to periodically report to FDA on the status of any study or clinical trial otherwise undertaken to investigate a safety issue. Section 506B of the FDCA, as well as 21 CFR 314.81(b)(2)(vii) requires you to report annually on the status of any postmarketing commitments or required studies or clinical trials.

FDA will consider the submission of your annual report under section 506B and 21 CFR 314.81(b)(2)(vii) to satisfy the periodic reporting requirement under section 505(o)(3)(E)(ii) provided that you include the elements listed in 505(o) and 21 CFR 314.81(b)(2)(vii). We remind you that to comply with 505(o), your annual report must also include a report on the status of any study or clinical trial otherwise undertaken to investigate a safety issue. Failure to submit an annual report for studies or clinical trials required under 505(o) on the date required will be considered a violation of FDCA section 505(o)(3)(E)(ii) and could result in enforcement action.

**POSTMARKETING COMMITMENTS SUBJECT TO REPORTING REQUIREMENTS OF SECTION 506B**

We remind you of your postmarketing commitment:

1895-9 To provide a detailed analysis of the effectiveness and safety of ELELYSO (taliglucerase alfa) for injection for 36 months obtained in the clinical development program compared with data available for the same length of treatment for other approved enzyme replacement therapies (ERT) for Gaucher disease.

The timetable you agreed to on April 26, 2012, states that you will conduct this study according to the following schedule:

Final Report Submission: 05/2013

**POSTMARKETING COMMITMENTS NOT SUBJECT TO REPORTING REQUIREMENTS OF SECTION 506B**

We remind you of your postmarketing commitments:

1895-10 To revise the cellular uptake potency assay release and stability acceptance criteria after 15 lots of drug product have been manufactured.

The timetable you agreed to on April 26, 2012, states that you will conduct this study according to the following schedule:

Final Report Submission: 07/2015

1895-11 To revise Experion automated electrophoresis release and stability acceptance criteria after 15 lots of drug product have been manufactured.

The timetable you agreed to on April 26, 2012, states that you will conduct this study according to the following schedule:

Final Report Submission: 07/2015

1895-12 To evaluate and revise as appropriate the minimal percentage of specific uptake of reference standard as a system suitability criterion in the cellular uptake potency assay after at least 80 independent assay runs of release and stability testing of drug substance and drug product lots have been completed.

The timetable you agreed to on April 26, 2012, states that you will conduct this study according to the following schedule:

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|---|---|
| Study Completion: | 12/2013 |
| Final Report Submission: | 03/2014 |

1895-13 To perform a thorough biochemical characterization of the [redacted] detected in the imaging capillary electrophoresis (iCE) assay and to evaluate the impact of this heterogeneity on product quality, including any effects on potency (specific uptake, enzyme kinetics, and cellular uptake). The characterization should use additional analytical assays (e.g., peptide mapping and [redacted]) to confirm the identity of the characterized peaks. Perform an assessment regarding the suitability and the implementation of the iCE method and other analytical assays as appropriate in your stability protocol. The results of these studies should guide the revision of the release and stability specifications after at least 30 lots of drug substance and at least 15 lots of drug product have been manufactured.

The timetable you agreed to on April 26, 2012, states that you will conduct this study according to the following schedule:

| | |
|---|---|
| Study Completion: | 04/2015 |
| Final Report Submission: | 07/2015 |

Submit clinical protocols to your IND 069703 for this product. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to this NDA. In addition, under 21 CFR 314.81(b)(2)(vii) and 314.81(b)(2)(viii) you should include a status summary of each commitment in your annual report to this NDA. The status summary should include expected summary completion and final report submission dates, any changes in plans since the last annual report, and, for clinical studies/trials, number of patients entered into each study/trial. All submissions, including supplements, relating to these postmarketing commitments should be prominently labeled "**Postmarketing Commitment Protocol,**" "**Postmarketing Commitment Final Report,**" or "**Postmarketing Commitment Correspondence.**"

## PROMOTIONAL MATERIALS

You may request advisory comments on proposed introductory advertising and promotional labeling. To do so, submit, in triplicate, a cover letter requesting advisory comments, the proposed materials in draft or mock-up form with annotated references, and the package insert to:

Food and Drug Administration
Center for Drug Evaluation and Research
Office of Prescription Drug Promotion
5901-B Ammendale Road
Beltsville, MD 20705-1266

As required under 21 CFR 314.81(b)(3)(i), you must submit final promotional materials, and the package insert, at the time of initial dissemination or publication, accompanied by a Form FDA 2253. For instruction on completing the Form FDA 2253, see page 2 of the Form. For more information about submission of promotional materials to the Office of Prescription Drug Promotion (OPDP), see http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm090142.htm.

**REPORTING REQUIREMENTS**

We remind you that you must comply with reporting requirements for an approved NDA (21 CFR 314.80 and 314.81).

**MEDWATCH-TO-MANUFACTURER PROGRAM**

The MedWatch-to-Manufacturer Program provides manufacturers with copies of serious adverse event reports that are received directly by the FDA. New molecular entities and important new biologics qualify for inclusion for three years after approval. Your firm is eligible to receive copies of reports for this product. To participate in the program, please see the enrollment instructions and program description details at http://www.fda.gov/Safety/MedWatch/HowToReport/ucm166910.htm.

**POST-ACTION FEEDBACK MEETING**

New molecular entities and new biologics qualify for a post-action feedback meeting. Such meetings are used to discuss the quality of the application and to evaluate the communication process during drug development and marketing application review. The purpose is to learn from successful aspects of the review process and to identify areas that could benefit from improvement. If you would like to have such a meeting with us, call the Regulatory Project Manager for this application.

If you have any questions, call Jessica Benjamin, Regulatory Project Manager, at (301) 796-3924.

Sincerely,

*{See appended electronic signature page}*

Julie Beitz, M.D.
Director
Office of Drug Evaluation III
Center for Drug Evaluation and Research

ENCLOSURE(S):
Content of Labeling
Carton and Container Labeling

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**This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.**

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/s/

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JULIE G BEITZ
05/01/2012

# EXHIBIT H

| Correspondence Date | Correspondence between Protalix Ltd. and FDA regarding Elelyso | Type of Correspondence |
|---|---|---|
| 2004 | | |
| June 30, 2004 | Pre-IND Meeting between FDA and Protalix | Meeting |
| July 28, 2004 | Correspondence from FDA to Protalix regarding Pre-IND meeting minutes | Letter |
| 2005 | | |
| June 15, 2005 | Correspondence from Protalix to FDA submitting IND | Submission |
| June 16, 2005 | Correspondence from FDA to Protalix regarding receipt of IND | Letter |
| July 15, 2005 | Communication from FDA to Protalix regarding clinical hold placed on IND | Letter |
| 2006 | | |
| November 29, 2006 | Type C meeting between FDA and Protalix | Meeting |
| December 21, 2006 | Correspondence from FDA to Protalix regarding Type C meeting minutes | Letter |
| 2007 | | |
| February 21, 2007 | Type B meeting between FDA and Protalix to discuss clinical hold | Meeting |
| March 22, 2007 | Correspondence from FDA to Protalix regarding Type B meeting minutes | Letter |
| March 28, 2007 | Correspondence from Protalix to FDA regarding special protocol assessment request for Trial PB-06-001 | Submission |
| April 16, 2007 | Communication from FDA to Protalix regarding removal of clinical hold placed on IND | Letter |
| May 11, 2007 | Correspondence from FDA to Protalix regarding clarification of protocol | Letter |
| August 22, 2007 | Correspondence from FDA to Protalix regarding acceptance of revised study designs | Letter |
| 2008 | | |
| April 14, 2008 | Type C meeting between FDA and Protalix | Meeting |
| 2009 | | |
| May 7, 2009 | Correspondence from FDA to Protalix regarding Type C meeting minutes | Letter |
| May 21, 2009 | Pre-NDA meeting between FDA and Protalix | Meeting |
| July 22, 2009 | Correspondence from Protalix to FDA submitting expanded access treatment protocol | Submission |
| August 14, 2009 | Communication from FDA to Protalix regarding decision to proceed with Trial PB-06-004 | Letter |
| August 24, 2009 | Communication from FDA to Protalix | Letter |

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| | granting Fast Track designation for investigation of taliglucerase alfa for the treatment of Type 1 Gaucher disease | |
| September 3, 2009 | Communication from FDA to Protalix granting orphan designation for the treatment of Type 1 Gaucher disease | Letter |
| September 14, 2009 | Correspondence from Protalix to FDA submitting NDA as rolling review | Submission |
| December 9, 2009 | Correspondence from Protalix to FDA submitting final section of NDA | Submission |
| December 9, 2009 | Correspondence from FDA to Protalix regarding incomplete NDA application | Letter |
| 2010 | | |
| January 5, 2010 | Correspondence from FDA to Protalix regarding NDA submission | Letter |
| January 7, 2010 | Correspondence from FDA to Protalix regarding NDA submission | Letter |
| January 25, 2010 | Correspondence from FDA to Protalix regarding request for information necessary to complete NDA review | Letter |
| January 28, 2010 | Correspondence from FDA to Protalix regarding NDA submission | Letter |
| March 3, 2010 | Correspondence from FDA to Protalix regarding NDA submission | Letter |
| April 26, 2010 | Correspondence from Protalix to FDA submitting NDA 022458 | Submission |
| April 28, 2010 | Correspondence from FDA to Protalix regarding receipt of NDA submission | Letter |
| April 30, 2010 | Correspondence from Protalix to FDA regarding assessment of proposed proprietary name | Submission |
| May 4, 2010 | Correspondence from Protalix to FDA regarding amendment to NDA | Submission |
| May 7, 2010 | Correspondence from FDA to Protalix regarding inquires for NDA submission | Email |
| May 26, 2010 | Meeting between FDA and Protalix regarding NDA submission | Meeting |
| June 1, 2010 | Communication from Protalix to FDA regarding teleconference for NDA clinical and biostatistical data | Email |
| June 2, 2010 | Teleconference between Protalix and FDA regarding NDA clinical and biostatistical data | Teleconference |
| June 4, 2010 | Correspondence from FDA to Protalix regarding proprietary name | Letter |
| June 4, 2010 | Correspondence from Protalix to FDA regarding corrected Debarment Certification | Submission |

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| June 7, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| June 7, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| June 11, 2010 | Correspondence from Protalix to FDA regarding corrected form FDA 356h | Submission |
| June 11, 2010 | Correspondence from FDA to Protalix regarding data definition files of NDA submission | Email |
| June 18, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| June 30, 2010 | Correspondence from Protalix to FDA regarding corrected form FDA 356h | Submission |
| July 9, 2010 | Correspondence from FDA to Protalix regarding NDA submissions | Letter |
| July 21, 2010 | Correspondence from Protalix to FDA regarding resubmission of portions of NDA | Submission |
| July 22, 2010 | Correspondence from FDA to Protalix regarding proprietary name | Letter |
| August 3, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| August 11, 2010 | Correspondence from FDA to Protalix regarding statistical and quality sections of NDA submission | Letter |
| August 13, 2010 | Correspondence from FDA to Protalix regarding proposed labeling | Letter |
| August 20, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| September 8, 2010 | Correspondence from FDA to Protalix regarding quality section of NDA submission | Letter |
| September 10, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| September 27, 2010 | Correspondence from Protalix to FDA regarding assessment of proposed proprietary name | Submission |
| October 1, 2010 | Correspondence from Protalix to FDA | Submission |
| October 28, 2010 | Correspondence from FDA to Protalix regarding information request for chemistry, manufacturing, and controls | Letter |
| November 8, 2010 | Correspondence from Protalix to FDA regarding genomic sequencing data | Email |
| November 10, 2010 | Correspondence from Protalix to FDA regarding genomic sequencing data | Submission |
| November 16, 2010 | Teleconference between Protalix and FDA regarding assessment of proposed proprietary name | Teleconference |

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| November 17, 2010 | Teleconference between Protalix and FDA regarding genomic sequencing data | Teleconference |
| November 19, 2010 | Correspondence from FDA to Protalix regarding cross-reactivity and confirmatory assays | Email |
| November 19, 2010 | Correspondence from FDA to Protalix regarding information request for chemistry, manufacturing, and controls | Letter |
| November 23, 2010 | Correspondence from FDA to Protalix regarding regarding PK information requests | Email |
| November 24, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| November 30, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| December 2, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| December 3, 2010 | Correspondence from Protalix to FDA regarding assessment of proposed proprietary name | Submission |
| December 10, 2010 | Correspondence from Protalix to FDA regarding assessment of proposed proprietary name | Submission |
| December 13, 2010 | Correspondence from FDA to Protalix regarding proprietary name | Email |
| December 20, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| December 21, 2010 | Correspondence from Protalix to FDA regarding PK information requests | Submission |
| December 22, 2010 | Correspondence from FDA to Protalix regarding regarding PK information requests | Email |
| December 23, 2010 | Correspondence from Protalix to FDA regarding label submission | Submission |
| December 27, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| December 28, 2010 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| 2011 | | |
| January 21, 2011 | Correspondence from FDA to Protalix regarding proprietary name | Letter |
| January 24, 2011 | Correspondence from FDA to Protalix regarding NDA submission | Letter |
| February 1, 2011 | Correspondence from FDA to Protalix regarding proprietary name | Letter |
| February 21, 2011 | Correspondence from FDA to Protalix regarding Complete Response Letter | Letter |
| May 3, 2011 | Type A meeting between FDA and Protalix | Meeting |

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| May 12, 2011 | Correspondence from FDA to Protalix regarding minutes of Type A meeting | Letter |
| July 31, 2011 | Correspondence from Protalix to FDA regarding Class 2 resubmission/Complete Response | Submission |
| August 1, 2011 | Receipt of Class 2 resubmission/Complete Response by FDA | Letter |
| August 8, 2011 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| August 17, 2011 | Teleconference between Protalix and FDA regarding US distribution of product | Teleconference |
| September 15, 2011 | Correspondence from FDA to Protalix regarding study site verification | Letter |
| September 23, 2011 | Correspondence from FDA to Protalix regarding Study PB-006-03 | Email |
| September 27, 2011 | Teleconference between Protalix and FDA regarding neutralizing anti-prGCD Antibodies Assay | Teleconference |
| October 5, 2011 | Correspondence from Protalix to FDA regarding API manufacturer | Submission |
| October 7, 2011 | Correspondence from Protalix to FDA regarding immunogenicity confirmatory assays | Letter |
| October 26, 2011 | Correspondence from FDA to Protalix regarding neutralizing anti-prGCD Antibodies Assay | Letter |
| October 31, 2011 | Correspondence from FDA to Protalix regarding immunogenicity confirmatory assays | Letter |
| November 1, 2011 | Correspondence from Protalix to FDA regarding clinical pharmacology amendment | Email |
| November 10, 2011 | Correspondence from FDA to Protalix regarding NDA submission | Letter |
| November 15, 2011 | Correspondence from Protalix to FDA regarding solicited major amendment | Submission |
| November 15, 2011 | Correspondence from Protalix to FDA regarding clinical pharmacology amendment | Submission |
| November 28, 2011 | Correspondence from FDA to Protalix regarding proprietary name | Letter |
| December 1, 2011 | Correspondence from FDA to Protalix regarding clinical pharmacology amendment | Letter |
| December 6, 2011 | Correspondence from FDA to Protalix regarding proprietary name | Letter |
| December 13, 2011 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| 2012 | | |

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|---|---|---|
| January 17, 2012 | Correspondence from FDA to Protalix regarding PK/PD report | Email |
| January 20, 2012 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| January 23, 2012 | Correspondence from Protalix to FDA regarding label submission | Submission |
| February 9, 2012 | Correspondence from FDA to Protalix regarding proprietary name | Letter |
| February 10, 2012 | Correspondence from Protalix to FDA regarding label submission | Submission |
| March 1, 2012 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| March 21, 2012 | Teleconference between Protalix and FDA regarding chemistry review and facility review and inspection | Teleconference |
| March 22, 2012 | Correspondence from Protalix to FDA regarding facility review and inspection | Submission |
| March 27, 2012 | Correspondence from Protalix to FDA regarding chemistry amendment | Submission |
| April 2, 2012 | Correspondence from Protalix to FDA regarding chemistry amendment | Submission |
| April 11, 2012 | Correspondence from Protalix to FDA regarding label submission | Submission |
| April 12, 2012 | Correspondence from FDA to Protalix regarding product labeling | Letter |
| April 13, 2012 | Correspondence from FDA to Protalix regarding product labeling | Letter |
| April 17, 2012 | Teleconference between Protalix and FDA regarding chemistry review | Teleconference |
| April 18, 2012 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| April 19, 2012 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| April 20, 2012 | Correspondence from Protalix to FDA regarding NDA information request | Submission |
| April 25, 2012 | Correspondence from Protalix to FDA regarding chemistry amendment | Submission |
| April 25, 2012 | Correspondence from Protalix to FDA regarding further clinical studies | Submission |
| April 27, 2012 | Correspondence from Protalix to FDA regarding further clinical studies | Submission |
| May 1, 2012 | Approval letter for Elelyso issued by FDA | Letter |